CHARACTERIZATION OF BIOMARKERS OF THROMBOSIS IN COVID-19

BIOMARKERS OF COAGULATION, ENDOTHELIAL DYSFUNCTION, AND FIBRINOLYSIS IN PATIENTS WITH COVID-19

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LAY ABSTRACT

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is responsible for the coronavirus disease-2019 (COVID-19) pandemic. COVID-19 presents as a respiratory tract infection, with severe cases causing substantial damage to the lungs as well as its surrounding blood vessels. The cause of death in COVID-19 is thought to be a consequence of lung and multiple organ injury resulting from a hyper-immune response to the virus, leading to a vicious cycle of cell damage and hyperactive blood clotting. However, why some patients worsen over time that could potentially lead to death while others recover remains uncertain. Our study aims to measure the circulating levels of various proteins that are involved in the formation and removal of blood clots over the course of the disease. Identifying which of these proteins can predict those that are likely to become severely ill could lead to early intervention for better outcome and survival.

ABSTRACT

Immunothrombosis and coagulopathy in lung microvasculature may lead to lung injury and disease progression in severe COVID-19. However, the mechanism that leads to decompensation and death for some patients, thus delineating them from patients who recover and survive, is poorly understood. We aim to identify biomarkers of coagulation, endothelial function, and fibrinolysis that are associated with disease severity and may have prognostic potential.

Our study has explored four different cohorts: 1) our pilot cohort, 2) COVID-BEACONS study cohort, 3) CanCOV study cohort and 4) ACTIV-4A trial cohort. The patient plasmas from these samples were quantified using 1) ELISAs for plasminogen, soluble thrombomodulin (sTM), plasminogen activator inhibitor-1 (PAI-1), α2-antiplasmin (A2AP), thrombin antithrombin complex (TAT), D-dimer, thrombin-activatable fibrinolysis inhibitor (TAFI), and fibrinogen, and 2) in-house functional assays for clot lysis times and activated TAFI (TAFIa) levels. Biomarker values were log-transformed and linear mixed effects models were used to compare trajectories in ICU and ward patients compared to outpatients from date of symptom onset.

Our pilot cohort showed that sTM, PAI-1, plasminogen and clot lysis times have predictive ability for mortality. In order to validate these findings, we explored the other three cohorts. The COVID-BEACONS cohort confirmed that plasminogen is associated with death and showed that fibrinogen and TAFI levels also predicted mortality in the COVID-19 patients. In the CanCOV study cohort, D-dimer and sTM antigen levels showed the strongest associations with moderate and severe COVID-19 compared to mild disease.

PAI-1, plasminogen, TAFIa, and fibrinogen may additionally be useful in identifying patients who become critically ill. Lastly, the ACTIV-4A trial cohort showed an increase in PAI-1, sTM and TAFIa levels in COVID-19 outpatients, whereas a longitudinal decrease in the antigen levels of fibrinogen and A2AP was observed.

The data from the cohorts needs to be normalized using clinical data and symptom onset data, and then used to validate the findings of our pilot cohort. Once that is achieved, our study has the potential to identify biomarkers that can predict which patients are likely to be severe and worsen over time, which could lead to early treatment and better chance of survival.

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VWF	von Willebrand factor
ICU	Intensive care unit
GP	Glycoproteins
ADP	Adenosine diphosphate
TXA2	Thromboxane A2
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
PLC	Phospholipase C
HMWK	High molecular weight kininogen
TF	Tissue factor
PS	Phosphatidylserine
polyP	Polyphosphates
PK	Prekallikrein
GAGs	Glycosaminoglycans
PC	Protein C
AT	Antithrombin
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
RCL	Reactive center loop
TAT	Thrombin-antithrombin complex
EPCR	Endothelial cell protein C
T-TM	Thrombin-thrombomodulin complex
APC	Activated protein C
K1, K2, and K3	Kunitz-type inhibitor domain
t-PA	Tissue-type plasminogen activator
u-PA	Urokinase-type plasminogen activator
FDPs	Fibrin degradation products
A2AP	α_2-antiplasmin

PAI-1	Plasminogen activator inhibitor-1
TAFI	Thrombin-activatable fibrinolysis inhibitor
PAI-2	Plasminogen activator inhibitor-2
IL	Interleukin
NHP	Normal human plasma
IIa-TM	Thrombin-thrombomodulin complex
SARS-COV-2	Severe acute respiratory syndrome coronavirus-2
COVID-19	Coronavirus disease-2019
S	Spike
RBD	Receptor-binding domain
C	Carbon
N	Amino
ACE-2	Angiotensin converting enzyme 2
ANGII	Angiotensin II
VOCs	Variants of concern
ARDS	Acute respiratory distress syndrome
ALI	Acute lung injury
sTM	Soluble thrombomodulin
COVID-BEACONS	Comprehensive biomarker analysis for prediction of clinical course and patient treatment outcomes
CanCOV	Canadian COVID-19 Prospective Cohort Study
ACTIV-4A	Accelerating COVID-19 Therapeutic Interventions and Vaccines 4
MTB	Modified Tyrodes Buffer
ACD	Acid-citrate dextrose
PRP	Platelet rich plasma
ATIII	Antithrombin
ELISA	Enzyme-linked immunosorbent assay

DECLARATION OF ACADEMIC ACHIEVEMENT

Ganeem K. Juneja completed all experiments, contributed to conception and design of the studies, analyzed and interpreted the data.

Vanessa Sabourin performed all experiments, analyzed and interpreted the data.

Dr. Paul Y. Kim contributed to the conception and design of the studies, obtained funding to support the studies, and critically reviewed and interpreted the obtained results.

Dr. Matthew Castelo and Dr. Bettina E. Hansen performed statical analyses on the data.

CHAPTER 1: INTRODUCTION

1.1 Overview of Hemostasis

Hemostasis involves a complex network of tightly regulated processes to rapidly respond to vessel injury through formation of fibrin clots, while maintaining blood in a fluid state where the circulation is intact (Gale, 2011; Versteeg et al., 2013). This system is required for maintaining the integrity of the circulatory system through a balance between procoagulant, anticoagulant, and fibrinolytic processes (Periayah et al., 2017). Upon vascular injury, subendothelial components in the vessel wall are exposed to circulating blood, triggering the activation of primary, secondary, and tertiary hemostasis (Periayah et al., 2017; Repetto & De Re, 2017).

1.1.1 Primary Hemostasis

Platelets are small anuclear cell fragments that bud off from megakaryocytes, specialized large polyploid blood cells that originate in the bone marrow (Schulze & Shivdasani, 2005). Platelets are present at 150 to 400 million per milliliter of blood and circulate for about ten days (Hoffman et al., 2013). In a healthy blood vessel, when there is normal blood flow, platelets do not adhere to surfaces or aggregate with each other. However, following vascular injury, platelets are exposed to subendothelial matrix, and adhesion, activation and aggregation of platelets begins to form a platelet plug; a process referred to as primary hemostasis (Repetto & De Re, 2017).

After an injury, the underlying subendothelial matrix components such as collagen, laminin, and von Willebrand factor (VWF) become exposed to the circulation (Wang et al., 2016). Using various adhesion molecules or glycoproteins (GP), circulating platelets

rapidly adhere to the damaged subendothelium (Ni & Freedman, 2003). For example, the GPIb-IX-V complex on platelets binds to the A1 domain of VWF, and the platelet receptors GPVI and integrin $\alpha_2\beta_1$ mediates binding with collagen (Ni & Freedman, 2003; Repetto & De Re, 2017). Once platelets adhere to the subendothelial matrix, they become activated and release many agonists, such as adenosine diphosphate (ADP) and thromboxane A2 (TXA2) (Estevez & Du, 2017).

Platelets have granules that store and release contents that are essential for normal platelet function (Blair & Flaumenhaft, 2009). There are the alpha granules which are most abundant granules, and contain several proteins including P-selectin, fibrinogen and VWF (Blair & Flaumenhaft, 2009). Whereas the dense granules contain molecules including ADP, adenosine triphosphate (ATP), polyphosphates, and calcium (Ca^{2+}) (Chen et al., 2018). The release of contents from these platelet granules results in further platelet activation, as well as platelet aggregation through several signaling mechanisms (Jackson, 2007).

The released agonist, from activated platelets, interact with platelet receptors. These receptors are G coupled proteins, such as Gq which activates phospholipase C (PLC) (Shah et al., 2001). This activation leads to the generation of second messengers, which stimulate protein kinase C (PKC), thus causing increased intracellular Ca^{2+} levels with the final effect of platelet aggregation (Durrant et al., 2017; Ley et al., 2016). Fibrinogen binds to $\alpha_{IIb}\beta_3$, which is a highly expressed integrin, forms bridges between adjacent platelets, thereby causing platelets to aggregate (Ley et al., 2016). As a result, a

platelet plug is formed which temporarily seals an injury in the vessel wall (Durrant et al., 2017; Ley et al., 2016).

1.1.2 Secondary Hemostasis: The Coagulation Cascade

The coagulation cascade is composed of series of sequential activation of zymogens to enzymes which ultimately leads to the cleavage of prothrombin to thrombin. Thrombin then converts soluble fibrinogen to insoluble fibrin clots (Versteeg et al., 2013). This process is dived into the extrinsic, intrinsic, and the common pathways (Figure 1) (Owens & Mackman, 2010; Versteeg et al., 2013). The coagulation cascade and fibrinolytic system are closely regulated to ensure a delicate balance in blood flow under normal physiological conditions; as excessive clotting can lead to thrombosis, whereas impaired clot formation, stability, or premature clot degradation can lead to hemorrhage (Pryzdial et al., 2018).

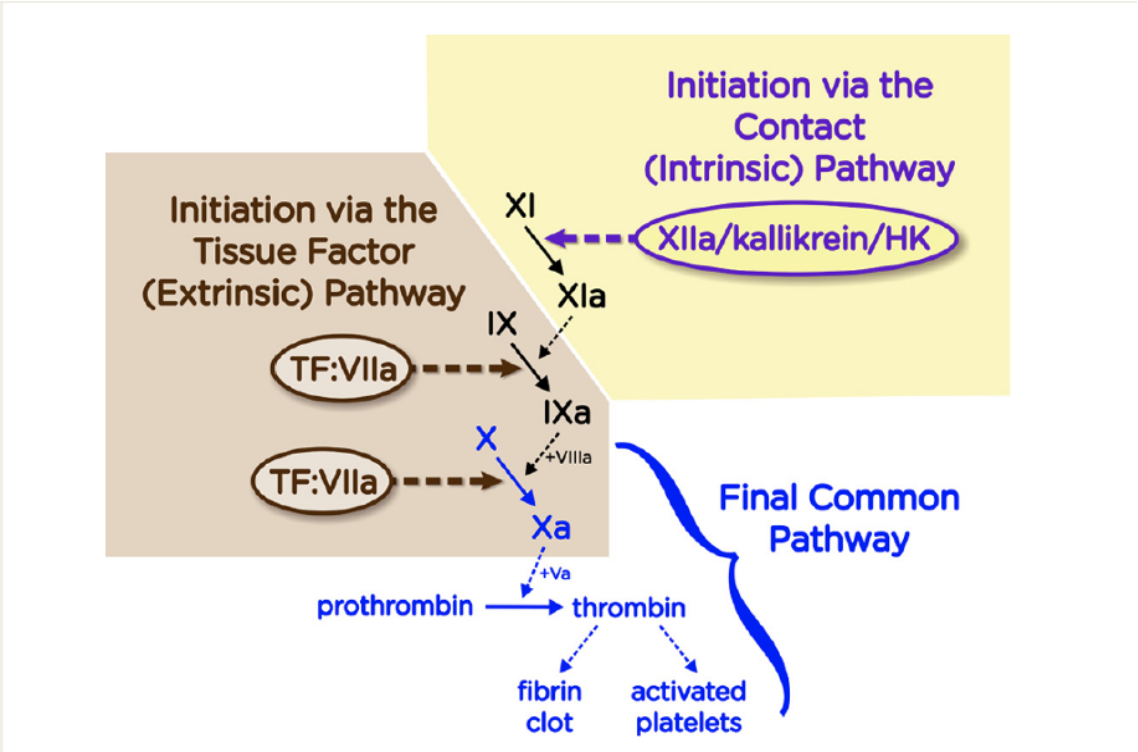

FIGURE 1: Overview of the coagulation cascade.

Upon initiation of the intrinsic/contact pathway, exposure to negatively charged surfaces generates factor (F) XIIa, kallikrein, and high molecular weight kininogen (HMWK/HK). This causes the activation of FXI to FXIa, which then activates FIX to FIXa. FIXa then binds with its cofactor FVIIIa on a negatively charged lipid surface to form the intrinsic tenase, which activates FX to FXa. Upon initiation of the extrinsic/TF pathway, co-localization of TF occurs, thus activating FIX to FIXa and FX to FXa. The common endpoint of both pathways is the formation of FXa, which interacts with its cofactor FVa on a negatively charged lipid surface to form the prothrombinase complex. Prothrombinase cleaves prothrombin to its active enzyme thrombin. Thrombin forms a stable clot by platelet activation and fibrin polymerization/deposition. (Obtained from (Smith et al., 2015)

1.1.3 The Extrinsic Pathway

The extrinsic pathway is initiated by the formation of the tissue factor (TF) and coagulation factor (F) VII complex (Monroe & Hoffman, 2006). TF, also known as thromboplastin, is a 47 kDa glycosylated, integral-membrane protein that is expressed by extravascular cells such as fibroblasts and vascular smooth muscle cells (Drake et al., 1989; Wilcox et al., 1989). When vascular injury occurs, subendothelial TF is exposed to the blood and binds to FVII and activated FVII (FVIIa). TF acts as a cofactor by promoting proteolysis and activation of FVII to FVIIa (Yau et al., 2015). Furthermore, the TF/FVIIa complex is also known as the extrinsic tenase complex, which in the presence of Ca^{2+} ions and phosphatidylserine (PS) efficiently activates FIX, a part of the intrinsic pathway, (Haynes et al., 2012; Mackman et al., 2007) and FX, which is the common pathway (Monroe & Hoffman, 2006).

1.1.4 The Intrinsic Pathway

The intrinsic pathways also known as the contact pathway, is initiated by exposure of blood to negatively charged surfaces (Colman & Schmaier, 1997). Examples of non-physiological activators include kaolin, glass, and silica and physiological activators includes collagen, polyphosphates (polyP), and nucleic acids (Colman & Schmaier, 1997). This exposure leads to the activation of the zymogen FXII into the serine protease FXIIa (Smith et al., 2015). FXIIa, along with its cofactor high-molecular-weight kininogen (HMWK), activates prekallikrein (PK) to plasma kallikrein (Smith et al., 2015). Plasma kallikrein forms a positive feedback loop by causing further activation of FXII (Muller et

al., 2011). Next, FXIIa in a complex with cofactor HMWK, activates its substrate FXI to FXIa, and FXIa activates FIX to FIXa (Gailani & Renne, 2007b). FIXa then binds with its cofactor FVIIIa to form the intrinsic tenase complex. This then leads to additional generation of FXa (Wu, 2015). A series of activation steps in each pathway ultimately leads to the convergence of both pathways at FX activation, which is the beginning of the common pathway.(Zhao et al., 2015).

1.1.5 The Common Pathway

The intrinsic and extrinsic pathways merge at the common pathway (Palta et al., 2014). The common pathway begins when FX becomes activated to FXa by either the extrinsic or intrinsic tenase complex (Palta et al., 2014; Smith et al., 2015). A prothrombinase complex is formed when FXa associates with its cofactor, FVa, on a negatively charged phospholipid surface (*i. e.* PS) in the presence of Ca^{2+} ions (Lechtenberg et al., 2013). This complex leads to the conversion of prothrombin into thrombin (Standeven et al., 2002). Thrombin then converts fibrinogen into fibrin monomers, and activates FXIII to FXIIIa (Standeven et al., 2002). Fibrin monomers polymerize into oligomers that lengthen and eventually aggregate into fibrin strands (Chernysh et al., 2012). FXIIIa covalently crosslinks the fibrin strands to form a stable and insoluble fibrin clot (Palta et al., 2014; Smith et al., 2015). In addition, thrombin activates FV, FVIII, and FXI, which further amplifies thrombin generation (Narayanan, 1999).

1.1.6 Regulation of the Coagulation Cascade

Naturally occurring anticoagulant in the body exert a regulatory role over the coagulation cascade to preventing systemic clotting and keeping the growing thrombi localized at the site of injury (Sira & Eyre, 2016). In a healthy vasculature, the endothelium is naturally anticoagulant to maintain the fluidity of blood. Major natural anticoagulant systems present in circulation include antithrombin (AT), protein C (PC), and tissue factor pathway inhibitor (TFPI) (Kubier & O'Brien, 2012; Periayah et al., 2017).

AT is a plasma glycoprotein that belongs to the serine protease inhibitor (serpin) family (Bock et al., 1982). It is produced by the liver and circulates in plasma at a concentration of 2.5 μM (Heit, 2013). It has a molecular mass of 58 kDa and a half-life of 3 days (Heit, 2013; Roemisch et al., 2002). AT possesses two specific active binding sites including a heparin-binding domain and a reactive center loop (RCL) (Perry, 1994). The RCL allows AT to bind and inhibit thrombin, FVIIa, FIXa, FXa, FXIa, and FXIIa while the heparin binding domain allows AT to bind to heparin (Perry, 1994). In the presence of glycosaminoglycans (GAGs) such as heparin or heparan sulfate, AT activity is increased more than 1000-fold (Tollefsen et al., 1983). When AT binds to thrombin, it forms the thrombin-antithrombin complex (TAT), which is used as an indicator for levels of thrombin that was generated in the blood.

Another major regulator of the coagulation pathway is the PC pathway (Esmon, 2003). This pathway has several important components including thrombin, thrombomodulin (TM), the endothelial cell protein C receptor (EPCR), PC, and protein S (Esmon, 2003). PC circulates in plasma at a concentration of 70 nM, has a molecular mass of 62 kDa, and a half-life of 10 hours in circulation (Gruber & Griffin, 1992; Kisiel, 1979;

Okajima et al., 1990). The PC pathway is initiated when thrombin binds to TM on endothelial cells and forms the thrombin-thrombomodulin complex (T-TM) (Dahlback, 2005). PC binds to EPCR and presents PC to the T-TM complex, thereby increasing the rate of activated protein C (APC) production by 20 fold (Dahlback, 2005; Esmon, 2003). APC then inactivates FVa and FVIIIa by proteolysis, a process that can be enhanced by the presence of protein S (Griffin et al., 2007).

TFPI is a Kunitz-type protease inhibitor that is produced by platelets and endothelial cells. TFPI exists in two major isoforms, TFPIα and TFPIβ, due to alternative splicing (Wood et al., 2014). It circulates in plasma at a concentration of 1.6 nM (Dahm et al., 2003; Maroney & Mast, 2008). It has a molecular mass of 43 kDa and a circulating half-life of 60-120 minutes (Broze & Girard, 2012; Lwaleed & Bass, 2006). TFPIα has three Kunitz-type inhibitor domains (K1, K2, and K3), while TFPIβ has the K1 and K2 domains (Mast, 2016; Wood et al., 2014). The K1 domain binds FVIIa, and the K2 domain binds FXa, thereby forming a TF-FVIIa-FXa-TFPI quaternary complex (Maroney & Mast, 2008; Wood et al., 2014). Thus, both TFPI isoforms inhibit the TF:FVIIa complex in a FXa-dependent manner (Mast, 2016). In contrast, only TFPIα inhibits the prothrombinase complex by binding of its basic C-terminal region to the B domain of FV, and its K2 domain to FXa (Wood et al., 2014).

Thus, the generation of procoagulant factors leading to clot formation is matched by equivalent proportions of anticoagulants, with sufficient capacity to establish a balance. Any defects within these inhibitory pathways will alter the balance that eventually leads to thrombotic tendencies (Periayah et al., 2017).

1.1.7 Tertiary Hemostasis: Fibrinolysis

Fibrinolysis (Figure 2) is the process of degrading the insoluble fibrin clots using plasmin, resulting in the cleavage of fibrin to form fibrin degradation products (FDPs). These FDPs are then readily cleared out of circulation. The smallest species of FDPs is a D-dimer, which provides an index of both coagulation and fibrinolysis. Plasmin is the central fibrinolytic enzyme and is generated from plasminogen on the surface of a fibrin clot. This is accomplished by the serine proteases, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Urano et al., 2018). t-PA is secreted constitutively by vascular endothelial cells and has a high affinity for plasminogen. u-PA is synthesized by monocytes, macrophages, and urinary epithelium, and typically functions in extravascular locations (Gailani & Renne, 2007b). t-PA activates plasminogen to plasmin, resulting in fibrin digestion. Although t-PA is a weak activator of plasminogen in the absence of fibrin, its catalytic efficiency is increased by a 1000-fold in the presence of fibrin (Cesarman-Maus & Hajjar, 2005). This is because fibrin acts as a cofactor in plasmin generation, thus aiding in its own degradation. Once formed, plasmin cleaves fibrin at specific arginine and lysine residues, generating soluble FDPs. The proteolytic cleavage results in generation of new C-terminal lysine residues, which then act as additional binding sites for plasminogen and t-PA (Cesarman-Maus & Hajjar, 2005; Chapin & Hajjar, 2015). This mediates further binding of plasminogen and t-PA to fibrin, leading to enhanced plasmin generation and fibrin degradation. Due to the continuous cleavage of fibrin and the generation of new C-terminal lysine residues, plasmin augments its own formation through

a positive feedback, which further accelerates overall fibrinolysis (Cesarman-Maus & Hajjar, 2005).

1.1.8 Inhibitors of Fibrinolysis

Fibrinolysis is regulated by 1) inhibitors that target plasminogen activators or plasmin, and 2) down-regulation of plasmin generation (Gailani & Renne, 2007a). Plasmin and plasminogen activators are neutralized by serpins, which form covalent complexes with their target enzymes and subsequently clear them from circulation. The three main serpins involved are α_2-antiplasmin (A2AP), plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2) (Bannish et al., 2017). The main down-regulator of fibrinolysis is thrombin-activatable fibrinolysis inhibitor (TAFI) (Bannish et al., 2017; Hoylaerts et al., 1982).

A2AP is a 464-amino acid single-chain glycoprotein that is the main physiologic inhibitor of plasmin (Rijken & Lijnen, 2009). A2AP is a serine protease inhibitor secreted by the liver, that crosslinks to both fibrinogen and fibrin during clot formation and protects the clot from fibrinolysis (Rijken & Lijnen, 2009). In circulation, A2AP is both in free form and bound to plasminogen and fibrinogen. It down-regulates fibrinolysis in three ways. First, A2AP forms a stoichiometric complex with plasmin (plasmin-antiplasmin complex) where unbound plasmin is inhibited more readily compared with clot-bound plasmin (Carpenter & Mathew, 2008). Second, it protects the fibrin clot from degradation by plasmin as it is crosslinked to both the precursor fibrinogen and fibrin by FXIIIa (Carpenter & Mathew, 2008). Third, by forming a complex with the lysine-binding sites of

plasminogen through its C-terminus, the binding of plasminogen to fibrin is inhibited, resulting in down-regulation of plasminogen activation (Carpenter & Mathew, 2008).

Originating from endothelial cells, PAI-1 is a 47 kDa 379-amino acid single chain glycoprotein member of the superfamily of serpins whose antigen circulates between 6 and 80 ng/mL and acts as the chief inhibitor of t-PA and u-PA (Cesari et al., 2010; Yasar Yildiz et al., 2014). PAI-1 is an important inhibitor of fibrinolysis as it directly inhibits t-PA, which ultimately inhibits plasmin generation. Expression of PAI-1 can be increased by several proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor alpha (TNFα) (Swiatkowska et al., 2005). PAI-2 serves a similar function; however, its concentration increases during pregnancy and deficiencies in it have been linked with unfavourable pregnancy outcomes (Bannish et al., 2017; Gould et al., 2015).

Fibrinolysis is down-regulated by TAFI, a 60 kDa procarboxypeptidase that is produced and secreted into circulation by the liver. It can be converted to its active form, TAFIa, by thrombin, T-TM, and plasmin (Butt & Swaminathan, 2008). TAFIa cleaves C-terminal lysine and arginine residues on fibrin, effectively reducing the number of plasminogen and t-PA binding sites that are present. This slows plasmin generation, thus stabilizing clots and attenuating fibrinolysis (Mosnier & Bouma, 2006).

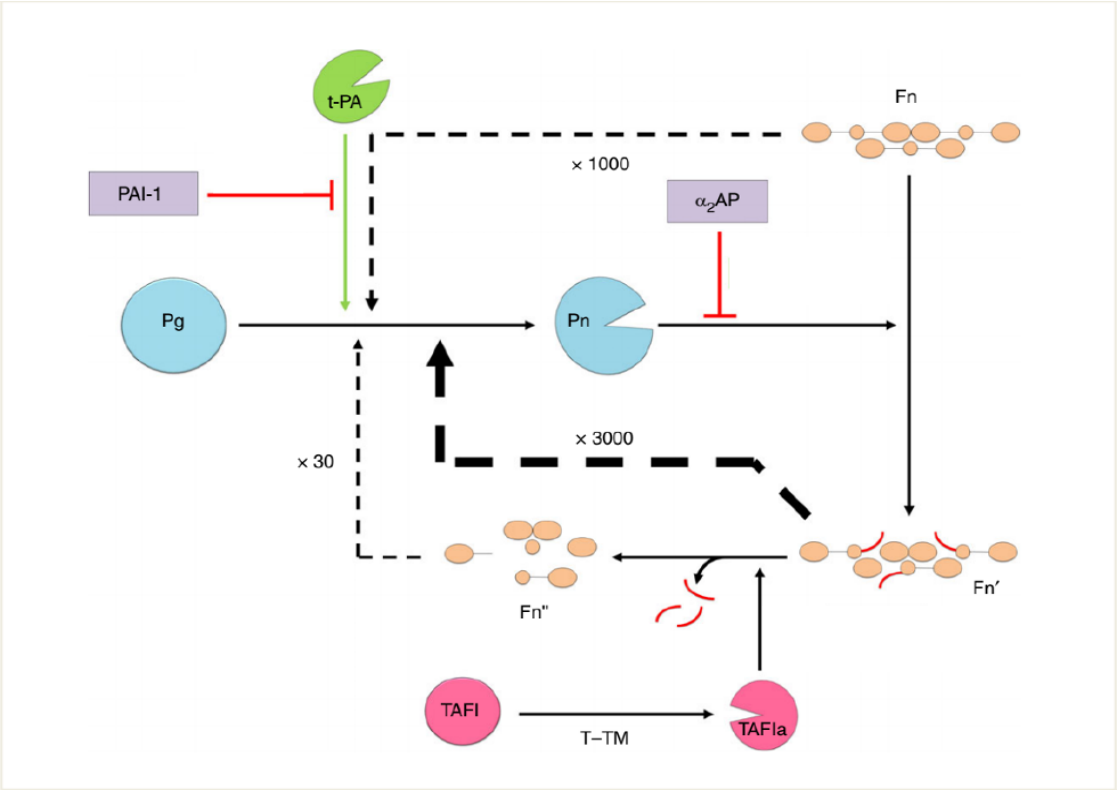

FIGURE 2: The fibrinolytic system.

t-PA, derived from endothelial cells, performs the cleavage of plasminogen to its activated form plasmin; the inhibitor PAI-1 regulates this process. The presence of a fibrin clot accelerates the formation of plasmin by 1000-fold. Plasmin cleaves fibrin, producing plasmin-modified fibrin which is 3-times as efficient as fibrin as a co-factor for plasmin generation. This is due to the newly exposed C-terminal lysine residues. A2AP is another inhibitor which functions by inhibiting plasmin, thus prolonging the lysis of clots. The T-TM, present on the endothelial cell surface, activates TAFI to TAFIa, which reduces the co-factor activity of plasmin-modified fibrin by removing the lysine residues. This generates TAFIa-modified fibrin (Henderson et al., 2018).

1.2 COVID-19

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the virus responsible for the coronavirus disease-2019 (COVID-19) pandemic. It is a newly emergent zoonotic virus that originated from Wuhan, China (Guan et al., 2020). First reported in December 2019, COVID-19 has spread to over 220 countries and territories, infecting over 535 million people worldwide and killing over 6.21 million as of June 10, 2022 (Zheng et al., 2020). This disease has soon become the most consequential global health crisis since the influenza pandemic of 1918 and overwhelmed the healthcare systems worldwide (Cascella et al., 2022). Even though substantial progress in clinical research has led to a better understanding of SARS-CoV-2 and the management of COVID-19, which has limited the spread of this virus and its variants, it still continues to wreak havoc across the world with many countries enduring third or fourth waves of outbreaks (Aleem et al., 2022).

1.2.1 SARS-CoV-2

Structurally and phylogenetically, SARS-CoV-2 is composed of four main structural proteins: spike (S), envelope glycoprotein, nucleocapsid, membrane protein, along with 16 non-structural proteins, and 5-8 accessory proteins (Jiang et al., 2020). The surface S glycoprotein is located on the outer surface of the virion and undergoes cleavage into an amino (N)-terminal S1 subunit, which facilitates the incorporation of the virus into the host cell and a carboxyl (C)-terminal S2 subunit which is responsible for virus-cell membrane fusion (Du et al., 2009; Jiang et al., 2020). The S1 subunit is further

divided into the N-terminal domain and receptor-binding domain (RBD), which is implicated in facilitating viral entry into the host cell and serves as a potential target for neutralization in response to vaccines (Song et al., 2018).

1.2.2 SARS-CoV-2 Pathogenesis

SARS-CoV-2 targets the angiotensin converting enzyme 2 (ACE-2) receptor and heparan sulfate on the surface of alveolar endothelial cells, binding through S proteins present on the viral envelope (Clausen et al., 2020; Connors & Levy, 2020; Hoffmann et al., 2020; Lu et al., 2020). ACE2 receptors play a vital role in regulating processes such as blood pressure, wound healing and inflammation, called the renin-angiotensin-aldosterone system pathway (Ni et al., 2020). They do this by modulating the function of the protein angiotensin II (ANG II) that increases blood pressure and inflammation, increasing damage to blood vessel linings and various types of tissue injury (Ni et al., 2020). When the SARS-CoV-2 virus binds to ACE2, it causes the subsequent internalization of the virus which diminishes the remaining membrane-bound ACE-2 receptors, potentially leading to increased angiotensin II levels in the lung (Malha et al., 2020).

The viral attachment process is followed by priming the S protein by the host transmembrane serine protease 2 that facilitates cell entry and subsequent viral replication endocytosis with the assembly of virions (Hoffmann et al., 2020). Two phases explain the pathogenesis of this virus. The first or the early phase is characterized by viral replication which is followed by the second or the late phase when infected host cells result in an immune response leading to the recruitment of T lymphocytes, monocytes, and neutrophil

recruitment which releases cytokines (*e.g* TNFα, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), interleukin-6 (IL-6), and interferon (IFN)-γ). This results in the overactivation of the immune system results in a cytokine storm characterized by the release of high levels of cytokines, especially IL-6 and TNF-α, into the circulation, causing a local and systemic inflammatory response (Azkur et al., 2020; Wang et al., 2020). The inflammation and endothelial damage leads to the activation of the clotting cascade as well as the dysfunction of the fibrinolytic system. This imbalance leads to clotting within the lungs that leads to hypoxemia and lung failure and subsequent downstream multiorgan failure (Figure 3) (Yeh et al., 2020).

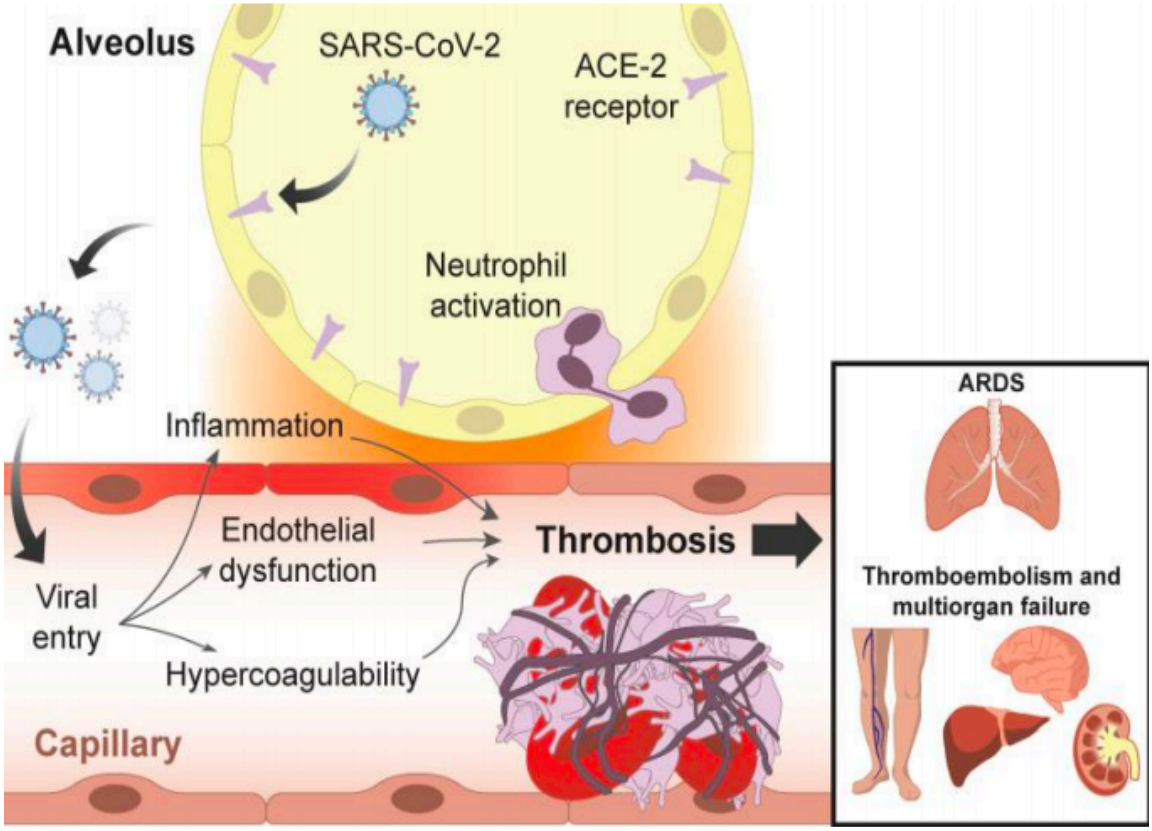

FIGURE 3: COVID-19 Pathogenesis.

The SARS-CoV-2 virus directly enters alveolar epithelial cells via the ACE-2 receptors. Infected epithelial cells generate a dysregulated inflammatory response due to viral exposure and it results in a cytokine storm, which leads to cell damage, largely endothelial damage. The inflammation and endothelial damage taken together leads to the activation of the clotting cascade as well as the dysfunction of the fibrinolytic system. This imbalance leads to clotting within the lungs that leads to hypoxemia and lung failure and subsequent downstream multiorgan failure (Yeh et al., 2020).

1.2.3 SARS-CoV-2 Variants

SARS-CoV-2, like other RNA viruses, is prone to genetic evolution while adapting to their new human hosts with the development of mutations over time, resulting in the emergence of multiple variants that may have different characteristics compared to its ancestral strains (Lauring & Hodcroft, 2021). Due to this, multiple variants of SARS-CoV-2 have been described, of which a few are considered variants of concern (VOCs) due to their potential to cause enhanced transmissibility or virulence, reduction in neutralization by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutics or vaccination effectiveness (Aleem et al., 2022; Cascella et al., 2022). The five main VOCs are alpha, beta, gamma, delta and omicron.

Alpha Variant

In late December 2020, the alpha variant of SARS-CoV-2 was reported in the UK based on whole-genome sequencing of samples from patients who tested positive for SARS-CoV-2 (Galloway et al., 2021; Volz et al., 2021). This variant included seventeen mutations, out of which eight were in the S protein (Wu et al., 2021). This variant was shown to have an increased affinity to the ACE2 receptors, enhancing viral attachment and subsequent entry into host cells (Davies et al., 2021; Walensky et al., 2021).

Beta Variant

Another variant of SARS-CoV-2 referred to as the beta variant was first reported in South Africa in October 2020 (Tegally et al., 2021). It has nine mutations in the S protein, three of which are located in the RBD. These changes in the RBD increase the binding affinity of this variant to the ACE receptors (Mwenda et al., 2021; Wibmer et al., 2021). This variant is also reported to have an increased risk of transmission and reduced neutralization by monoclonal antibody therapy, convalescent sera, and post-vaccination sera (Wang et al., 2021).

Gamma Variant

The third variant of concern is the gamma variant, which was first reported December 2020 in Brazil and was first detected in the USA in January 2021 (Faria et al., 2021). This variant harbors ten mutations in the S protein and three of these mutations are located in the RBD (Faria et al., 2021).

Delta Variant

The fourth variant of concern is the delta variant and it was initially reported in December 2020 in India. This variant also has ten mutations in the S proteins (Aleem et al., 2022).

Omicron Variant

Omicron variant is the fifth variant of concern and it was first identified in South Africa in November 2021(Vaughan, 2021). This variant has 30 mutations in the S protein which has an overwhelmingly disruptive effect, which makes this variant more likely to have vaccine breakthroughs (Chen et al., 2022). This variant is the most transmissible variant to date for all age group.

1.2.4 COVID-19 Coagulopathy

COVID-19 presents as a lower respiratory tract infection with severe cases progressing similarly to acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) pathophysiology, whereby intravascular and extravascular fibrin deposition occurs, increasing the risk of mortality (Gattinoni et al., 2020; Yuki et al., 2020). ARDS entails an increase in alveolar permeability in response to infection, leading to pulmonary edema and acute hypoxemia, with severe cases requiring invasive mechanical ventilation (Matthay et al., 2019). Fibrin deposition is a result of increased coagulability and decreased fibrinolysis at the site of infection, which has also been reported in COVID-19 patients via thromboelastographic analysis (Camprubí-Rimblas et al., 2018; Wright et al., 2020; Xu et al., 2020). This hypercoagulable state typically presents with increased circulating levels of fibrinogen, FVIII, D-dimer, and VWF (Yeh et al., 2020). Additionally, platelets have recently been shown to exhibit hyperreactivity in response to infection, with increased circulating platelet-neutrophil and platelet-monocyte aggregates in severe patients (Manne et al., 2020). Platelets were also shown to induce tissue factor expression in monocytes upon infection, with some associating and even internalizing COVID-19 RNA (Manne et

al., 2020; Zaid et al., 2020). Additionally, a recent study reported the presence of ACE-2 on the surface of platelets in critically ill patients, possibly explaining increased platelet hyperreactivity, inflammation, and associated thrombotic risk in COVID-19 patients (L. Zhang et al., 2020).

COVID-19 patients admitted to the intensive care unit (ICU) are administered thromboprophylactic agents, however up to 30% of ICU patients and 16% of all hospitalized COVID-19 patients experience thrombotic complications, including increased risk for arterial and/or venous thrombosis, pulmonary embolism, ischemic stroke, and myocardial infarction (Bilaloglu et al., 2020; Moll et al., 2020; Yeh et al., 2020). COVID-19 patients are at higher risk of thrombotic complications due to the resulting inflammatory response, platelet hyperreactivity, and endothelial dysfunction (Bikdeli et al., 2020). Conversely, fibrinolysis is also reportedly affected, with both thromboelastography (TEG) and rotational thromboelastometry (ROTEM) illustrating fibrinolysis inhibition in COVID-19 patients (Nougier et al., 2020; Seheult et al., 2020). However, patients exhibit markedly increased levels of D-dimer, prothrombin time, and activated partial thromboplastin time, which may also correlate with poor outcomes (Colling & Kanthi, 2020; Long et al., 2020; Tang et al., 2020; S. Zhang et al., 2020). As D-dimer is a degradation product of fibrin clots, the cause of substantial levels in COVID-19 patients remains unclear, with some suggesting the role of microparticles, neutrophils, or a local hyperfibrinolytic state at various stages of infection that cannot be detected using whole blood assays (Colling & Kanthi, 2020; Seheult et al., 2020).

Critically-ill COVID-19 patients require intensive hospitalized intervention, where some respond to treatments and recover, and others decompensate and may succumb to infection (Kermali et al., 2020; Zeng et al., 2020). As fibrinolysis is reportedly inhibited in critical COVID-19 patients, it currently remains unknown how COVID-19 infection alters fibrinolytic processes to allow for widespread clotting fibrin deposition. Both t-PA and PAI-1 are released by endothelial cells in response to inflammation, with activated platelets also releasing large amounts of PAI-1, this leads to a hypofibrinolytic environment (Nougier et al., 2020). A recent report noted increased levels of t-PA, PAI-1, and TAFIa in severe COVID-19 cases, indicating fibrinolysis prolongation through inhibition of plasmin activation (Nougier et al., 2020). However, blood for this study was collected from COVID-19 patients within the first 3 days of hospital admission. As the infection progresses, insights into the impairment of fibrinolysis over time may provide more reliable and effective biomarkers to better determine prognosis and subsequent clinical course of the infection.

1.3 Objectives, Hypothesis and Significance

1.3.1 Objectives

The primary objective of this study is to identify biomarkers of coagulation, fibrinolysis and endothelial dysfunction that may predict clinical course and outcome in COVID-19 patients.

1.3.2 Hypothesis

A deviation in the biomarker levels of coagulation, fibrinolysis and endothelial dysfunction in COVID-19 patients can be used to predict disease state with prognostic value.

1.3.3 Significance

The COVID-19 pandemic is far from over and future spikes have the potential to kill millions more. These bursts of critically-ill patients are the main reason why our healthcare systems have been overwhelmed for the past two years. Early treatment of COVID-19 patients as well as early identification of those at high risk of decompensation may lead to improved outcome and decreased burden in our healthcare system.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study Design

This study consisted of four different cohorts: 1) Pilot study, 2) COVID-BEACONS (comprehensive biomarker analysis for prediction of clinical course and patient treatment outcomes) study, 3) The Canadian COVID-19 Prospective Cohort Study (CanCOV; NCT05125510), and 4) The Accelerating COVID-19 Therapeutic Interventions and Vaccines 4 (ACTIV-4A; NCT04505774) trial.

2.1.1 Pilot study cohort

Consecutive patients ≥18 years of age admitted to the ICU meeting clinical criteria for suspected COVID-19 were prospectively enrolled and analyzed. Patients were included from March 16, 2020 to April 24, 2020, corresponding to the initial COVID-19 outbreak in our health region. If not known at clinical presentation, COVID-19 status was confirmed by two positive polymerase chain reaction tests for the SARS-CoV-2 virus. Daily blood draws were initiated at the time of ICU admission and were continued up to day 3 for COVID-19(-) patients (on a ventilator in the ICU and tested negative for COVID-19) and up to day 10 for COVID-19(+) patients (on a ventilator in the ICU and tested positive for COVID-19). All COVID-19(+) patients were age- and sex-matched with a COVID-19(-) patient from the prospectively collected pool to form a critically ill control group. Previously collected blood samples from a healthy control group were assembled from age- and sex-matched participants held at the Translational Research Centre in London, Ontario.

All the patient and clinical characteristics between COVID-19(+) and COVID-19 (-) patients for this cohort are in Table 1.

TABLE 1: Patient and clinical characteristics between COVID-19(+) and age- and sex-matched COVID-19(-) critically ill patients in the pilot London cohort.

Patient characteristics	COVID-19(+) (n=14)	COVID-19(-) (n=14)
Age	61 (54 – 67)	58.5 (52.5 – 63)
Male	6 (43%)	6 (42.9%)
Comorbidities		
Diabetes	5 (35.7%)	5 (35.7%)
Hypertension	7 (50.0%)	9 (64.3%)
Coronary artery disease	2 (14.3%)	2 (14.3%)
Congestive heart failure	0	2 (14.3%)
Chronic kidney disease	2 (14.3%)	1 (7.1%)
Cancer	2 (14.3%)	1 (7.1%)
COPD	1 (7.1%)	3 (21.4%)
Chest x-ray findings		
Normal	0	3 (21.4%)
Unilateral pneumonia	1 (7.1%)	8 (57.1%)
Bilateral pneumonia	13 (92.9%)	2 (14.3%)
Interstitial/atypical findings	0	1 (7.1%)
MODS	4 (3 – 5.5)	6 (3 – 8)
SOFA	4.5 (2 – 9.25)	6 (4.25 – 10.5)
Mean arterial pressure	84 (72.75 – 97.5)	75.5 (59.25 – 107.25)
P/F ratio	107 (65.5 – 161.675)	172 (137.75 – 312)
WBC	8.45 (6.9 – 16.075)	15.25 (11.05 – 20.45)
Lymphocytes	0.7 (0.55 – 1)	1.3 (0.5 – 1.75)
Neutrophils	7.3 (5.6 – 12.55)	12.2 (8.1 – 15.725)
Lactate	1.5 (1 – 2)	1.2 (0.9 – 1.6)
Platelets	206 (133.5 – 293.75)	201.5 (163.75 – 259.5)
Hemoglobin	121.5 (101.5 – 134.5)	123.5 (101.75 – 137.75)
Creatinine	81.5 (57.5 – 187)	75 (54.25 – 113)
INR	1.2 (1.1 – 1.3)	1.05 (1 – 1.125)
aPTT	28 (25 – 31)	23 (19.5 – 25.25)
Treatments		
Antibiotics	14 (100%)	14 (100%)
Antivirals	3 (21.4%)	2 (14.3%)
Steroids	3 (21.4%)	5 (35.7%)
Vasoactive medications	11 (78.6%)	8 (57.1%)
Renal replacement therapy	2 (14.3%)	1 (7.1%)
Antiplatelet agent	5 (35.7%)	7 (50.0%)
Anticoagulation	13 (92.9%)	14 (100%)
Respiratory support		
High-flow nasal oxygen	8 (57.1%)	1 (7.1%)
NIMV	6 (42.9%)	8 (57.1%)
Invasive ventilation	10 (71.4%)	11 (78.6%)
Died	7 (50.0%)	2 (14.3%)

2.1.2 COVID-BEACONS study cohort

We are conducting a multi-center prospective longitudinal cohort study of ICU patients with a primary diagnosis of COVID-19 associated pneumonia. The inclusion criteria for this cohort is: 1) diagnosis or presumptive diagnosis of severe COVID-19 disease with respiratory compromise, 2) ventilation required, and 3) if transferred, admitted to local site within 72 hours of initial ICU admission. The exclusion criteria for this cohort is known or suspected pregnancy; patient, substitute-decision maker, or most responsible physician has not consented to study enrollment. Once a patient was enrolled, serial blood samples are collected from critically ill COVID-19 patients as they progress through their illness. Venous blood is collected from patients at the indicated time points: days 1, 3, 5, 7, 10, 14, 21, and 28. We have recruited a total of 49 patients from the Hamilton General Hospital site between February 2021 to April 2022, while 12 patients were recruited from the Kingston Health Science Center between September 2021 to April 2022. All patient and clinical characteristics of this cohort are in Table 2.

TABLE 2: Patient and clinical characteristics of the critically-ill COVID-19(+) patients in the COVID-BEACONS study cohort.

Patient Characteristics	COVID-19 (+) patients (n=21)
Age (median [IQR])	63.00 [54.00, 71.00]
Sex (%)	
Female	6 (28.6)
Male	15 (71.4)
Comorbidities	
Diabetes (%)	8 (38.1)
Hypertension (%)	13 (61.9)
Coronary artery heart disease (%)	4 (19.0)
Chronic congestive heart failure (%)	2 (9.5)
Chronic kidney disease (%)	1 (4.8)
Cancer (%)	2 (9.5)
COPD (%)	1 (4.8)
Treatments	
Antibiotics (%)	20 (95.2)
Antivirals (%)	2 (9.5)
Steroids (%)	20 (95.2)
Vasoactive medications (%)	20 (95.2)
Renal replacement therapy (%)	1 (4.8)
High flow nasal cannula (%)	9 (42.9)
Invasive mechanical ventilation (%)	21 (100.0)
Anticoagulation (%)	21 (100)
Chest x-ray findings (%)	
Normal	0 (0)
Unilateral pneumonia	0 (0)
Bilateral pneumonia	20 (95.2)
Interstitial findings	1 (4.8)
P/F ratio (median [IQR])	85.00 [74.00, 145.70]
MODS (median [IQR])	6.00 [4.00, 8.00]
SOFA (median [IQR])	10.00 [9.00, 11.00]
ICU days (median [IQR])	19.00 [14.00, 29.00]
Died	11 (52.4)

2.1.3 CanCOV study cohort

Longitudinal samples were collected for patients with COVID-19 as part of the CanCOV study. There is a total of 99 patients in this cohort and it is divided into three different groups: outpatients, ward patients and ICU patients. The outpatient group, 23 patients, were the patients that tested positive for COVID-19 and were required to stay at home and isolate. The ward patients were sick enough to require admission to acute hospital care and there were 31 patients for this group. The last group is the ICU patient group, 45 patients, which are the patients that are critically ill and were in the ICU or required mechanical ventilation. Venous blood was collected from patients for minimum 1 and up to 5 timepoints. All patient and clinical characteristics that is available for this cohort are in Table 3.

TABLE 3: Patient and clinical characteristics of the critically-ill COVID-19(+) patients in the CanCOV study cohort. The missing clinical data is indicated in the

Characteristic	Overall, n = 120[1]	Deceased, n = 31	Hosp, n = 33	ICU, n = 33	Outpatient, n = 23
Age	56 (47, 67)	58 (50, 68)	65 (54, 87)	57 (50, 65)	37 (31, 46)
Sex					
Female	57 (48%)	13 (42%)	19 (58%)	12 (36%)	13 (57%)
Male	63 (52%)	18 (58%)	14 (42%)	21 (64%)	10 (43%)
Comorbidities					
Hypertension	36 (43%)	1 (50%)	22 (67%)	12 (46%)	1 (4.3%)
Missing	36	29	0	7	0
Cardiovascular disease	41 (49%)	1 (50%)	25 (76%)	14 (54%)	1 (4.3%)
Missing	36	29	0	7	0
Diabetes	16 (19%)	2 (100%)	8 (24%)	6 (23%)	0 (0%)
Missing	36	29	0	7	0
Liver disease	2 (2.4%)	0 (0%)	2 (6.1%)	0 (0%)	0 (0%)
Missing	36	29	0	7	0
Malignancy	7 (8.3%)	0 (0%)	6 (18%)	1 (3.8%)	0 (0%)
Missing	36	29	0	7	0
Kidney disease					
Mild CKD	1 (1.2%)	0 (0%)	1 (3.0%)	0 (0%)	0 (0%)
Moderate/severe CKD	5 (6.0%)	0 (0%)	3 (9.1%)	2 (7.7%)	0 (0%)
No	78 (93%)	2 (100%)	29 (88%)	24 (92%)	23 (100%)
Missing	36	29	0	7	0
Stroke	2 (2.6%)	0 (0%)	0 (0%)	2 (7.7%)	0 (0%)
Missing	42	30	5	7	0
Treatments					
Antivirals	21 (30%)	0 (NA%)	12 (48%)	9 (38%)	0 (0%)
Missing	49	31	8	9	1
Antibiotics	30 (41%)	0 (NA%)	13 (50%)	16 (64%)	1 (4.5%)
Missing	47	31	7	8	1
Steroids	44 (58%)	0 (NA%)	24 (86%)	20 (77%)	0 (0%)
Missing	44	31	5	7	1
Anticoagulation	7 (11%)	3 (9.7%)	0 (NA%)	4 (12%)	0 (NA%)
Missing	56	0	33	0	23

2.1.4 ACTIV-4A trial cohort

This cohort consists of 91 patients and is part of the ACTIV trial which is investigating an approach to prevent clotting events and improving outcomes in COVID-19 patients. This trial has already been tested in critically ill COVID-19 patients in the ICU (R.-C. Investigators et al., 2021) as well as noncritically ill COVID-19 ward patients (A. Investigators et al., 2021). These two studies showed that a therapeutic-dose of anticoagulation with heparin did not result in a greater probability of survival or decreased severity in ICU patients (R.-C. Investigators et al., 2021). However, a therapeutic-dose of anticoagulation with heparin increased the probability of survival and reduced the use of cardiovascular or respiratory organ support in the noncritically ill ward patients (A. Investigators et al., 2021).

The samples we received from this trial are of outpatients with COVID-19 who are given a therapeutic-dose of anticoagulation with heparin to prevent or reduce the formation of blood clots in and improve the outcomes of these patients. Venous blood was collected from patients on day 0, 3, 7 and 14. All patient and clinical characteristics that is available for this cohort are in Table 4.

TABLE 4: Patient and clinical characteristics of the critically-ill COVID-19(+) patients in the ACTIV-4A trial cohort. The missing clinical data is indicated in the table.

Characteristic	Overall, n= 91[1]	Alive, n = 72[1]	Died, n = 19[1]
Age	60 (50, 67)	59 (49, 65)	65 (58, 69)
Sex			
Female	38 (42%)	28 (39%)	10 (53%)
Male	53 (58%)	44 (61%)	9 (47%)
Comorbidities	47 (52%)	32 (44%)	15 (79%)
Hypertension	51 (96%)	38 (95%)	13 (100%)
Missing	38	32	6
Heart failure	4 (7.5%)	3 (7.5%)	1 (7.7%)
Missing	38	32	6
Myocardial infarction	3 (5.7%)	2 (5.0%)	1 (7.7%)
Missing	38	32	6
Coronary artery disease	8 (15%)	6 (15%)	2 (15%)
Missing	38	32	6
Stroke	3 (5.7%)	2 (5.0%)	1 (7.7%)
Missing	38	32	6
Malignancy	1 (20%)	1 (25%)	0 (0%)
Missing	86	68	18
Autoimmune	1 (20%)	0 (0%)	1 (100%)
Missing	86	68	18
Diabetes	31 (94%)	24 (100%)	7 (78%)
Missing	58	48	10
Liver disease	3 (9.1%)	1 (4.2%)	2 (22%)
Missing	58	48	10
Asthma	19 (70%)	18 (78%)	1 (25%)
Missing	64	49	15
COPD	9 (33%)	6 (26%)	3 (75%)
Missing	64	49	15

2.2 Materials

Human alpha thrombin was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Normal human plasma (NHP) was obtained from healthy adult volunteers via venipuncture and processed as previously described.[12] Recombinant t-PA (Activase) was purchased from Kingston General Hospital Pharmacy (Kingston, ON, Canada). Prostaglandin (PG) I_2 was purchased from Sigma Aldrich (Oakville, Ontario). Fibrinogen, plasminogen, TAT and TAFI enzyme-linked immunosorbent assay (ELISAs) were purchased from Affinity Biologicals (Ancaster, ON, Canada). PAI-1 and A2AP ELISAs were purchased from Molecular Innovations (Novi, MI, USA). D-Dimer ELISA was purchased from RayBiotech (Peach Corners, GA, USA). Soluble thrombomodulin (sTM) ELISA was purchased from R&D systems (Minneapolis, MN, US). Antithrombin (ATIII) and ATIII deficient plasma was purchased from Affinity Biologicals (Ancaster, ON, Canada). COVID-19 patient blood samples for COVID-BEACONS study will be obtained from two sites: Hamilton General Hospital in Hamilton, Ontario and Kingston Health Science Centre in Kingston, Ontario. The COVID-19 patient plasma samples for the pilot study were obtained from Translational Research Centre in London, Ontario in collaboration with Dr. Douglas Fraser. Additional COVID-19 patient plasma samples were obtained from the CanCOV study in collaboration with Drs. Angela Cheung and Margaret Herridge, and the ACTIV-4A trial in collaboration with Dr. Matthew Neal. Clot lysis experiments were performed using HBS (0.02 M HEPES, 0.15 M NaCl, pH 7.4) with 0.01% Tween 80 (HBST). Acid citrate dextrose (ACD) and Modified Tyrodes buffer (MTB) was used for platelet isolation. All microtiter plates used were 96-well clear flat-

bottom plates pre-treated for at least 1 hour with HBS containing 1% Tween 80 (Sigma-Aldrich, Oakville, ON, Canada) and washed thoroughly with water and air dried prior to use. SpectraMax M2 plate reader (Molecular Devices, Sunnyvale CA, USA) was used to measure absorbance.

2.3 Methods

2.3.1 Biomarkers of Interest

COVID-19 patients have been reported to be in a hypercoagulable state with suppressed fibrinolysis; inflammation from the disease has also been shown to result in in endothelial dysfunction. Therefore, in order to identify biomarkers that predict clinical outcome in COVID-19 patients, we measured levels of those biomarkers that play a role in coagulation, fibrinolysis, and endothelial dysfunction. For coagulation, we measured levels of fibrinogen, TAT and D-dimer using an ELISA. To explore the impact COVID-19 has on fibrinolysis, we measured PAI-1, TAFI, A2AP, and plasminogen levels using ELISAs. Clot lysis times and TAFIa levels were measured using in-house functional assays. Lastly, levels of sTM were measured using and ELISA in order to investigate endothelial function in COVID-19 patients.

2.3.2 Sample processing for the COVID-BEACONS study cohort

After collection, processing of the blood should be done as soon as possible (within 2 hours) after collection in order to prevent the unwanted release of mediators caused by dying cells.

Plasma Processing

Each tube containing patient blood was inverted once to mix the contents. The tubes were placed in a swinging bucket centrifuge at 1,700 x g for 20 minutes at 4°C with deceleration set to low. The plasma was carefully collected using a Pasteur pipette by placing the tip at the meniscus and traveling down along with the meniscus. The plasma was then transferred into a new chilled tube. The plasma was centrifuged a second time at 2,500 x g for 20 min at 4°C with deceleration set to medium. The plasma was then aliquoted into corresponding cryogenic freezing tubes and stored in the -80°C freezer.

Platelet Isolation

First, 300 µL of prewarmed 10% Acid-citrate dextrose (ACD) solution was added to the tube containing patient blood. Then the tube was centrifuged in a swinging bucket centrifuge at 21°C, 200 x g and deceleration at 0-1. The sample was centrifuged for 20 minutes and then platelet rich plasma (PRP) is carefully removed from the sample. Then 4µL of 1 mM PGI_2 for every 1 mL of PRP is added to the sample and it is centrifuged again at 1000 x g for 10 minutes with a deceleration of 5. The plasma is then removed from the tube and aliquoted into its corresponding cryogenic freezing tubes and stored in the -80°C freezer. The pallet of platelets at the bottom of the tube was resuspended with 2x the original PRP volume of Modified Tyrodes Buffer (MTB) containing glucose. Then 0.12x the volume of MTB of ACD was added to the sample. Then 2 µL of 1mM PGI_2 was added for every 1 mL of PRP volume. Finally, the platelets were centrifuged one last time at 1000 x g for 10 minutes with a deceleration of 5.The supernatant is then removed from the sample and 0.5x the PRP volume of MTB is added. Then the platelets were counted using the

hemocytometer. The isolated platelets were viable for the next 5 hours at room temperature with minimal disruption.

2.3.3 Clot lysis

Clot lysis times are often used as the indicator of overall fibrinolytic potential. As such, it will be used as the main biomarker to infer inhibition of fibrinolysis. Patient plasma diluted 1:3 in HBST was added to the wells and clotting and lysis were initiated with 5 nM thrombin and 0.75 nM t-PA, respectively, in the presence of 10 mM $CaCl_2$, and absorbance was monitored at 405 nm for 2 h at 37°C at 15 s intervals using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). Clotting time and lysis time were determined as the time to reach half-maximal increase and decrease in absorbance as determined by the instrument software.

2.3.4 Functional TAFIa assay

While commercial antigen-based ELISAs are available to measure TAFI levels, there are no commercial assays readily available for quantifying levels of TAFIa in plasma (Foley et al., 2013) As such, we rely on our in-house assay for measuring TAFIa levels down to pM range (Kim et al., 2008). The assay relies on binding equilibrium between plasminogen labeled with a fluorescent probe and FDPs labeled with a quencher. Addition of plasma samples containing TAFIa will lead to removal of plasminogen binding C-terminal lysines on FDPs, which leads to dissociation of plasminogen from the FDPs. The

end result is increased fluorescence that is directly proportional to the concentration of TAFIa.

2.3.5 Clot Lysis with patient (COVID-19 patients, sepsis patients or healthy controls) platelets

NHP and patient plasma was diluted 1:3 in HBST with $1x10^8$ platelets/mL of patient platelets. The mixture was added to the wells and clotting and lysis were initiated with 5 nM thrombin and 0.75 nM t-PA, respectively, in the presence of 10 mM $CaCl_2$, and absorbance was monitored at 405 nm for 2 h at 37°C at 15 s intervals using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). Clotting time and lysis time were determined as the time to reach half-maximal increase and decrease in absorbance as determined by the instrument software.

2.3.6 ELISAs

The protocol provided by the manufacturer was followed for all the ELISAs we performed to measure antigen levels of our biomarkers of interest. For the ELISAs we either got antibody-coated plates in the ELISA kit or coated our own plates. The procedure for both ELISAs is as following, however the coating and blocking steps were not required for the ELISAs that came with antibody coated plates. Additionally, the procedure below

does not specify incubation times, dilutions and buffers used as they were followed directly from the manufacturer protocol for each ELISA.

Coating

Coating buffer was warmed to room temperature and the coating antibody was diluted into it. Once diluted, 100 µL of the solution was added to each well of the plate using a multichannel pipette. The plate was covered an incubated at room temperature.

Blocking

The plate was emptied and washed using a multichannel pipette. The plate was then dried using Kim wipes and 100 µL of blocking buffer was added to each well using a multichannel pipette. The plate was covered and incubated at room temperature.

Adding Samples

Plasma samples were defrosted at room temperature and diluted to achieve desired concentration. Then 100 µL of each diluted sample was added to the washed antibody-coated plate using a multichannel pipette. The plate is then covered and incubated at room temperature.

Detecting Antibody

The plate was emptied and washed again as before. 100 µL of diluted detecting antibody is added into each well using a multichannel pipette. The plate was covered and incubated at room temperature.

Chromogenic Substrate

The plate was emptied and washed as mentioned before. Chromogenic substrate mixture was freshly prepared, and 100 µL of the mixture was added into each well using a multichannel pipette. The colour was allowed to develop and then the stop solution was added.

Reading the Absorbance

The absorbance of the standard and samples was measured using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Standard Curve and Calculations

Average absorbance value of each set of duplicate standard was calculated then the standard curve was created by plotting mean absorbance (y axis) against the corresponding concentration (x axis). A curve or line of best fit is made for the curve using SigmaPlot and the generated equation is used to calculate the absorbance of the samples.

2.3.7 Preparing TAT complex standard reference

Purified ATIII (5 µM) in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 1 mM EDTA and 0.05 U/mL heparin, is incubated with a limiting amount of thrombin (1 µM) at 37^{o}C for 10 minutes. Complete inhibition should be confirmed by plasma clot time or chromogenic assay. If all thrombin is completely inhibited, the concentration of TAT complex is 1 µM.

CHAPTER 3.0: RESULTS

3.1 Determining the biomarkers levels for the pilot study cohort

Juneja, G. K., Castelo, M., Yeh, C. H., Cerroni, S. E., Hansen, B. E., Chessum, J. E., Abraham, J., Cani, E., Dwivedi, D. J., Fraser, D. D., Slessarev, M., Martin, C., McGilvray, S., Gross, P. L., Liaw, P. C., Weitz, J. I., & Kim, P. Y. (2021). Biomarkers of coagulation, endothelial function, and fibrinolysis in critically ill patients with Covid-19: A single-center prospective longitudinal study. *Journal of Thrombosis and Haemostasis*, *19*(6), 1546–1557. https://doi.org/10.1111/jth.15327

Author Contributions:

Douglas D. Fraser, Marat Slessarev, and Claudio Martin collected the patient samples and associated deidentified clinical data. Calvin H. Yeh, Scott McGilvray, Peter L. Gross, Patricia C. Liaw, Jeffrey I. Weitz, and Paul Y. Kim designed the experiments. Ganeem K. Juneja, Samantha E. Cerroni, James E. Chessum, Joel Abraham, Erblin Cani, and Douglas D. Fraser generated the data. Matthew Castelo, Bettina E. Hansen, and Paul Y. Kim analyzed the data.

Personal thesis contribution: As the co-primary author of the paper, I have provided most of the data and experimental analysis which contributed towards all the figures of this manuscript. In addition, I provided revision of the primary draft.

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ORIGINAL ARTICLE

Biomarkers of coagulation, endothelial function, and fibrinolysis in critically ill patients with COVID-19: A single-center prospective longitudinal study

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Abstract

Background: Immunothrombosis and coagulopathy in the lung microvasculature may lead to lung injury and disease progression in coronavirus disease 2019 (COVID-19). We aim to identify biomarkers of coagulation, endothelial function, and fibrinolysis that are associated with disease severity and may have prognostic potential.

Methods: We performed a single-center prospective study of 14 adult COVID-19(+) intensive care unit patients who were age- and sex-matched to 14 COVID-19(−) intensive care unit patients, and healthy controls. Daily blood draws, clinical data, and patient characteristics were collected. Baseline values for 10 biomarkers of interest were compared between the three groups, and visualized using Fisher's linear discriminant function. Linear repeated-measures mixed models were used to screen biomarkers for associations with mortality. Selected biomarkers were further explored and entered into an unsupervised longitudinal clustering machine learning algorithm to identify trends and targets that may be used for future predictive modelling efforts.

Results: Elevated D-dimer was the strongest contributor in distinguishing COVID-19 status; however, D-dimer was not associated with survival. Variable selection identified clot lysis time, and antigen levels of soluble thrombomodulin (sTM), plasminogen activator inhibitor-1 (PAI-1), and plasminogen as biomarkers associated with death. Longitudinal multivariate k-means clustering on these biomarkers alone identified two clusters of COVID-19(+) patients: low (30%) and high (100%) mortality groups. Biomarker trajectories that characterized the high mortality cluster were higher clot lysis times (inhibited fibrinolysis), higher sTM and PAI-1 levels, and lower plasminogen levels.

Conclusions: Longitudinal trajectories of clot lysis time, sTM, PAI-1, and plasminogen may have predictive ability for mortality in COVID-19.

Ganeem K. Juneja and Matthew Castelo contributed equally to this study.

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KEYWORDS
biomarkers, coronavirus, fibrinolysis, observational study, thrombosis

Essentials

- COVID-19 is an immunothrombotic disease that leads to respiratory failure.
- It is unknown why some critically ill patients recover while some decompensate.
- We identified four potential biomarkers of fibrinolysis and endotheliopathy associated with mortality.
- If validated, these biomarkers may have predictive ability for worse outcomes.

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which is responsible for the novel coronavirus disease 2019 (COVID-19) pandemic, is a newly emergent zoonotic coronavirus that appears to have originated in Wuhan, China.[1] First reported in December 2019, COVID-19 has spread to more than 200 countries and territories, infecting more than 94 million people worldwide and killing over 2 million.[2]

SARS-CoV-2 is thought to target the angiotensin converting enzyme 2 receptor and heparan sulfate on the surface of alveolar endothelial cells, binding through spike S proteins on the viral envelope.[3-6] Subsequent internalization of the virus diminishes remaining membrane-bound angiotensin converting enzyme 2 receptors, potentially leading to increased angiotensin II levels, further promoting hypoxemia and resulting lung injury.[7] Consequently, COVID-19 presents as a lower respiratory tract infection, with severe cases progressing to acute respiratory distress syndrome with intravascular and extravascular fibrin deposition.[8-11]

Increased rates of systemic thrombotic complications are a prominent feature in COVID-19 patients. Emerging observations suggest that the immunothrombotic responses to SARS-CoV-2 in the lung microvasculature may contribute to disease progression. Fibrin deposition in the lungs, elevated levels of D-dimer, and rates of thrombosis ranging from 5% to 30% despite thromboprophylaxis[12-16] are hallmarks of COVID-19 pneumonia.[17-19] Therefore, modulation of the immunothrombosis and coagulopathy may prevent lung injury in COVID-19.

Current evidence suggest dysregulation of the coagulation and fibrinolytic systems in COVID-19 patients.[20-29] Although the coagulation system has been studied extensively,[30,31] the fibrinolytic system has not been longitudinally evaluated in intensive care unit (ICU) patients with or without COVID-19. To address this gap, we explored the time course of markers of coagulation (fibrinogen, D-dimer, thrombin-antithrombin [TAT] complex), endothelial function (soluble thrombomodulin [sTM]), and fibrinolytic activity (plasminogen, plasminogen activator inhibitor-1 [PAI-1], plasmin-antiplasmin [PAP] complex, thrombin-activatable fibrinolysis inhibitor [TAFI], activated TAFI [TAFIa], clot lysis time) in patients with COVID-19 admitted to the ICU and associations with mortality that may inform future predictive modelling efforts.

2 | EXPERIMENTAL PROCEDURE

2.1 | Study design and setting

This was a prospective cohort study conducted at a single ICU at an academic tertiary care hospital in London, Canada.[32-36] The study was approved by the Western University Human Research Ethics Board and written informed consent obtained. We followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for cohort studies in the preparation of this manuscript.[37]

2.2 | Participants

Consecutive patients ≥18 years of age admitted to the ICU meeting clinical criteria for suspected COVID-19[38] were prospectively enrolled and analyzed. Patients were included from March 16, 2020, to April 24, 2020, corresponding to the initial COVID-19 outbreak in our health region. If not known at clinical presentation, COVID-19 status was confirmed by two positive polymerase chain reaction tests for the SARS-CoV-2 virus. Patients that subsequently tested negative for SARS-CoV-2 were maintained to form a COVID-19(–) critically ill control group. Thus, both groups initially met the same inclusion criteria at the time of ICU admission. Daily blood draws were initiated at the time of ICU admission and were continued up to day 3 for COVID-19(–) patients, and up to day 10 for COVID-19(+) patients. All COVID-19(+) patients were age- and sex-matched with a COVID-19(–) patient from the prospectively collected pool. Previously collected blood samples from a healthy control group were assembled from age- and sex-matched participants held at the Translational Research Centre in London, Ontario.[39,40]

2.3 | Clinical data

Baseline patient characteristics included age, sex, comorbidities, and presenting chest x-ray findings. Disease severity was classified using the Sequential Organ Failure Assessment (SOFA) and Multiple Organ Dysfunction scores (MODS). Both patient groups were characterized as having confirmed or suspected sepsis diagnosis using

Sepsis 3.0 criteria.[41] Clinical data were prospectively collected including the lowest or worst perfusion-ventilation (P/F) ratio, mean arterial pressure, and standard laboratory values. Recorded interventions included the use of antibiotics or antivirals, systemic corticosteroids, vasopressors, respiratory support, renal replacement, antiplatelet agents, and anticoagulants. Patients that survived to hospital discharge were considered survivors for the purposes of these analyses.

2.4 | Materials

Human tissue-type plasminogen activator (tPA; Alteplase) was purchased from Kingston General Hospital pharmacy. Human α-thrombin was from Enzyme Research Laboratories. A recombinant plasminogen derivative labeled with fluorescein and C_9-maleimide-QSY-labeled fibrin degradation products were prepared as described previously.[42] The human PAP complex ELISA kit was purchased from Biomatik. Human plasminogen, VisuLize TAFI, TAT complex, and fibrinogen antigen ELISA kits were from Affinity Biologicals Inc. The human PAI-1 total antigen ELISA kit was from Molecular Innovations. D-dimer was quantified using an ELISA kit from RayBiotech. sTM was quantified using the thrombomodulin/BDCA-3 Quantikine ELISA kit from R&D Systems. All of the assays were performed according to the manufacturers' protocols. The intra- and interassay variability of the commercial ELISAs are respectively as follows: PAP complex: 8% and 10%; TAFI: 5.8% and 8%; PAI-1: 5.9% and 6.5%; D-dimer: <12% and <10%; sTM: 2.9% and 6.9%; and fibrinogen: 9.4% and 13.4%. These values for plasminogen and TATs are unknown.

2.5 | Functional assays of fibrinolysis

Clot lysis assays were performed as described previously.[43] Briefly, 96-well clear flat-bottom microtiter plates were pretreated for at least 1 h with 0.02 M HEPES, 0.15 M NaCl, pH 7.4 (HBS) containing 1% Tween 80 (Sigma- Aldrich), and washed thoroughly with water. Plasma diluted 1:3 in HBS was added to the wells and clotting and lysis were initiated with 5 nM thrombin and 1 nM tPA, respectively, in the presence of 10 mM $CaCl_2$, and absorbance was monitored at 405 nm for 2 h at 37°C at 15-s intervals using a SpectraMax M2 plate reader (Molecular Devices). Clotting time and lysis time were determined as the time to reach half-maximal increase and decrease in absorbance as determined by the instrument software.

Functional levels of TAFIa were quantified as described previously.[42] Briefly, 50 µl of a solution containing 50 nM recombinant plasminogen derivative labeled with fluorescein and 100 nM C_9-maleimide-QSY-labeled fibrin degradation products in HBS was placed in wells of a 96-well white U-bottom microtiter plate. Baseline fluorescence readings were measured at 1-min intervals at 25°C, with excitation and emission wavelengths of 495 nm and 535 nm, respectively, with a cutoff at 515 nm. After equilibration, a plasma solution (50 µl) with a final 1:5 dilution was added. Reactions were monitored for 2 h and TAFIa levels were measured by quantifying rates of fluorescence increase.

2.6 | Statistical analysis

Baseline clinical characteristics were compared between COVID-19(+) patients and age- and sex-matched COVID-19(–) controls. Paired Wilcoxon rank-sum tests were used for continuous variables and Fisher's exact tests for categorical variables. Biomarkers determined on ICU admission day 1 (baseline) were compared in COVID-19(+) patients, COVID-19(–) patients, and healthy controls in a similar manner. Baseline biomarkers between the three groups were further visualized using Fisher's linear discriminant function, a nonparametric dimensionality reduction technique.[44] Before dimensionality reduction, biomarkers were normalized, and the results were displayed on a biplot.

Individual biomarker trajectories were plotted in COVID-19(+) patients, stratified by survivors and nonsurvivors. Variable selection for modelling was performed using individual repeated measures linear mixed models, comparing log-transformed biomarker values between survivors and nonsurvivors. Mean differences in biomarker values that reached our prespecified *p*-value cutoff of .25 were included, a typical threshold when performing *p*-value screening on a small dataset.[45] These biomarkers were further analyzed using a more complex mixed model by additionally including the day of measurement in the model and an interaction term between day of measurement and vital status, beginning at the first day of ICU admission. Where the interaction term was nonsignificant, and there was no apparent trajectory difference between groups on visual inspection, the interaction term was removed from the model. First-order autoregressive covariance structures were used for all mixed modelling.

Selected biomarkers were then included in a longitudinal, multivariate k-means clustering algorithm (*kml3d* package in R),[46,47] with the number of known clusters set at two. It was our *a priori* hypothesis that clustering may be able to divide the cohort into higher and lower disease severity clusters. Cluster sizes up to six were also tested and resulted in single membership in clusters, and lower performance scores (Calinski-Harabatz Index).[48] The machine learning algorithm was naïve to death status or any patient characteristic aside from the selected biomarkers. A sensitivity analysis that excluded one patient who died before day 10 did not change the results. Mean and individual biomarker values, and patient characteristics were described for the two clusters.

Missing biomarker data were imputed with linear interpolation, which is a more prudent method than mean imputation when the values before and after the missing value are known. All analyses were performed using SAS version 9.4 (SAS Institute Inc.) and RStudio (RStudio Inc.). All statistical tests were two-sided, and a *p*-value ≤.05 was considered statistically significant. Because this study was primarily exploratory and hypothesis-generating, adjustments for multiple comparisons were not made.[49]

TABLE 1 Patient and clinical characteristics between COVID-19(+) and age- and sex-matched COVID-19(–) critically ill patients

Patient Characteristics	COVID-19(+) (*n* = 14)	COVID-19(–) (*n* = 14)	*p* Value
Age, y	61 (54–67)	58.5 (52.5–63)	.4616
Male	6 (43%)	6 (42.9%)	1
Comorbidities			
Diabetes	5 (35.7%)	5 (35.7%)	1
Hypertension	7 (50.0%)	9 (64.3%)	.7036
Coronary artery disease	2 (14.3%)	2 (14.3%)	1
Congestive heart failure	0	2 (14.3%)	.4815
Chronic kidney disease	2 (14.3%)	1 (7.1%)	1
Cancer	2 (14.3%)	1 (7.1%)	1
COPD	1 (7.1%)	3 (21.4%)	.5956
Chest x-ray findings			
Normal	0	3 (21.4%)	<.001
Unilateral pneumonia	1 (7.1%)	8 (57.1%)	
Bilateral pneumonia	13 (92.9%)	2 (14.3%)	
Interstitial/atypical findings	0	1 (7.1%)	
MODS	4 (3–5.5)	6 (3–8)	.3262
SOFA	4.5 (2–9.25)	6 (4.25–10.5)	.1555
Mean arterial pressure	84 (72.75–97.5)	75.5 (59.25–107.25)	.7695
P/F ratio	107 (65.5–161.675)	172 (137.75–312)	.1514
WBC	8.45 (6.9–16.075)	15.25 (11.05–20.45)	.104
Lymphocytes	0.7 (0.55–1)	1.3 (0.5–1.75)	.03581
Neutrophils	7.3 (5.6–12.55)	12.2 (8.1–15.725)	.2734
Lactate	1.5 (1–2)	1.2 (0.9–1.6)	1
Platelets	206 (133.5–293.75)	201.5 (163.75–259.5)	1
Hemoglobin	121.5 (101.5–134.5)	123.5 (101.75–137.75)	.8752
Creatinine	81.5 (57.5–187)	75 (54.25–113)	.9442
INR	1.2 (1.1–1.3)	1.05 (1–1.125)	.04022
aPTT	28 (25–31)	23 (19.5–25.25)	.006323
Treatments			
Antibiotics	14 (100%)	14 (100%)	1
Antivirals	3 (21.4%)	2 (14.3%)	1
Steroids	3 (21.4%)	5 (35.7%)	.6776
Vasoactive medications	11 (78.6%)	8 (57.1%)	.4197
Renal replacement therapy	2 (14.3%)	1 (7.1%)	1
Antiplatelet agent	5 (35.7%)	7 (50.0%)	.7036
Anticoagulation	13 (92.9%)	14 (100%)	1
Respiratory support			
High-flow nasal oxygen	8 (57.1%)	1 (7.1%)	.01275
NIMV	6 (42.9%)	8 (57.1%)	.7064
Invasive ventilation	10 (71.4%)	11 (78.6%)	1
Died	7 (50.0%)	2 (14.3%)	.1032

Note: Values shown are median (interquartile range) and *N* (%). Statistical tests used were paired Wilcoxon rank-sum tests and Fisher's exact tests.

Abbreviations: COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 19; INR, international normalized ratio; MODS, Multi-organ Dysfunction Score; NIMV, noninvasive mechanical ventilation; P/F ratio, perfusion/ventilation ratio; aPTT, activated partial thromboplastin time; SOFA, sequential organ failure assessment score; WBC, white blood cell

3 | RESULTS

3.1 | Patient characteristics

Over the study period, 14 COVID-19(+) patients were identified in our ICU, as were age- and sex-matched with 14 COVID-19(−) critically ill patients and 14 healthy controls. Patient characteristics are presented in Table 1. Thirteen of the 14 COVID-19(+) patients received anticoagulation treatment with prophylactic dose low-molecular weight heparin (dalteparin; 5000 or 7500 units/day), whereas one patient received acetylsalicylic acid alone (81 mg/d). Four of the 13 patients treated with dalteparin also received acetylsalicylic acid (81 mg/d). Compared with COVID-19(−) patients, those with COVID-19 were significantly more likely to have bilateral pneumonia, lymphopenia, and require high-flow nasal oxygen. The COVID-19(+) cohort also had small but significant increases in international normalized ratio (INR) and activated partial thromboplastin time values. Although not statistically significant, mortality among COVID-19(+) patients was 50% (7/14) compared with 14.3% (2/14) in the critically ill controls.

3.2 | Biomarkers and COVID-19 status

The 10 biomarkers of interest at baseline are presented for each group in Table 2. Univariate comparisons between COVID-19(+) and (−) patients demonstrated no significant differences, except for D-dimer, which was higher in COVID-19(+) patients. Both groups had impaired clot lysis, with approximately one-half of the patients having lysis times above 100 mins. Compared with healthy controls, COVID-19(+) patients had significantly higher levels of PAI-1, sTM, D-dimer, fibrinogen, and TAT; lower levels of plasminogen and TAFI; and longer clot lysis times. Dimensionality reduction was performed using Fisher's linear discriminant function. Figure 1 shows a biplot with the patient groups plotted according to the first two linear discriminants, which explains 77.49% of the variance of the centroids. D-dimer was the strongest contributor toward identifying COVID-19 status, followed by sTM.

3.3 | Biomarkers and survival

Mortality among COVID-19(+) patients in our cohort was 50%, with follow-up extending until hospital discharge. Patient characteristics

TABLE 2 Biomarkers between COVID-19(+) patients, age- and sex-matched COVID-19(−) patients, and age- and sex-matched healthy controls

Biomarker at Baseline	COVID-19(+) (*n* = 14)	COVID-19(−) (*n* = 14)	Healthy Controls (*n* = 14)	COVID-19(+) to COVID-19(−) *p* Values	COVID-19(+) to Healthy Controls *p* Values
PAI-1 (ng/ml)	40.9 (30.7–56.5)	52.3 (21.3–90.2)	9.8 (0.7–14.4)	.391	<.001
Plasminogen (μM)	1.2 (1.1–1.4)	1.3 (1.1–1.5)	1.8 (1.4–2.0)	.2958	.01074
PAP (μg/ml)	0.7 (0.5–1.6)	0.6 (0.2–1.5)	0.8 (0.2–1.2)	.6257	.7609
TAFI (nM)	106.9 (77.1–115.0)	121.3 (100.2–132.0)	129.8 (118.8–175.2)	.1353	.008545
TAFIa (pM)	75.9 (23.4–124.3)	162.3 (67.0–217.4)	26.6 (0.0–87.3)	.1353	.2166
sTM (ng/ml)	5.1 (4.2–8.1)	5.7 (3.8–7.4)	3.3 (3.0–3.4)	1	.003052
D-dimer (μg/ml)	3.5 (2.5–5.4)	1.3 (0.9–1.7)	0.7 (0.6–0.8)	<.001	<.001
Fibrinogen (mg/ml)	10.7 (8.9–11.3)	10.3 (8.4–11.2)	7.1 (5.7–7.6)	.9032	.005249
Lysis time (min)					
<20	3 (21.4%)	2 (14.3%)	12 (85.7%)	.7844	.00226
20–59.9	4 (28.6%)	6 (42.9%)	2 (14.3%)		
60–99.9	0	0	0		
100+	7 (50.0%)	6 (42.9%)	0		
TAT (μg/L)	54.2 (38.8–143.5)	30.9 (19.8–98.6)	9.3 (6.3–30.5)	.5016	<.001

Note: Values shown are median (interquartile range) and *N* (%). Statistical tests used were paired Wilcoxon rank-sum tests and Fisher's exact tests.
Abbreviations: COVID-19, coronavirus disease 19; PAI-1, plasminogen activator inhibitor 1; PAP, plasmin-antiplasmin complex; sTM, soluble thrombomodulin; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated thrombin-activatable fibrinolysis inhibitor; TAT, thrombin-antithrombin complex

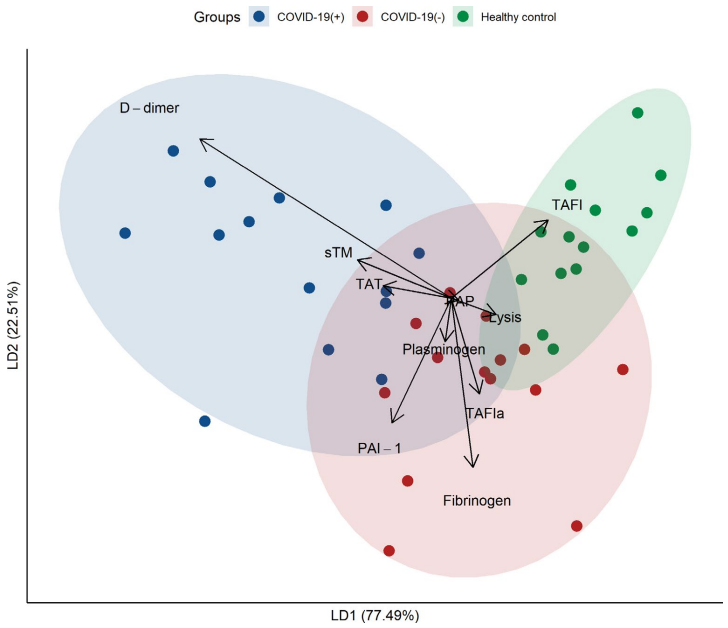


FIGURE 1 Biplot from Fisher's linear discriminant function, showing the three patient groups in two dimensions based on the baseline coagulopathy biomarkers. The first linear discriminant axis explains 77.49% of the variance of the centroids, on which D-dimer is the highest contributor. PAI-1, plasminogen activator inhibitor 1; PAP, plasmin-antiplasmin complex; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated thrombin-activatable fibrinolysis inhibitor; sTM, soluble thrombomodulin; TAT, thrombin-antithrombin complex

in survivors and nonsurvivors are presented in Table S1. As expected, nonsurvivors had trends toward more comorbidities and more abnormal laboratory values and were more likely to require invasive ventilation and vasoactive medications.

We explored the relationships between our 10 biomarkers of interest and survival. Individual trajectories for all biomarkers stratified by survivors and nonsurvivors are presented in Figure 2. After variable screening, four biomarkers that had associations with death that met the *p* value cutoff of <.25: clot lysis time, sTM, PAI-1, and plasminogen (Table S2). More complex repeated-measures mixed models were specified for these biomarkers (Figure 3). At baseline, there was a significant difference between survivors and nonsurvivors in the mean sTM values ($p = .0423$). There was a significant temporal trend among all COVID-19(+) patients for mean plasminogen ($p = .0003$) and sTM ($p < .0001$) values, with both biomarkers increasing over time. Individual interaction term parameters indicated there was significantly higher mean clot lysis time among nonsurvivors compared with survivors at day 10 ($p = .0041$). Although individual interactions were nonsignificant for day-to-day comparisons in plasminogen, the overall inclusion of an interaction was significant ($p = .0495$), suggesting the two groups had different trajectories.

These four biomarkers were then entered into an unsupervised longitudinal k-means clustering algorithm. This algorithm assigned the 14 COVID-19(+) patients into two clusters based only on the joint trajectories of the biomarkers (Figure 4). One cluster represented low mortality (cluster A; 30% [3/10]), whereas the other cluster represented high mortality (cluster B; 100% [4/4]). Patients in cluster B also had trends toward higher SOFA scores, MODS white blood cell count, and creatinine. This is consistent with creatinine being identified as a mortality predictor using metabolomics analysis.[34] More patients in cluster B required vasoactive medications and invasive ventilation. The biomarker trends that defined the severe disease/high mortality cluster were longer clot lysis times, higher levels of sTM and PAI-1, and lower levels of plasminogen.

4 | DISCUSSION

This initial, hypothesis-generating study is part of a larger effort to investigate the potential of markers of the coagulation and fibrinolytic systems to predict clinical courses and outcomes in critically ill COVID-19(+) patients (COVID-BEACONS [COVID-19: Comprehensive biomarker analysis for prediction of clinical course

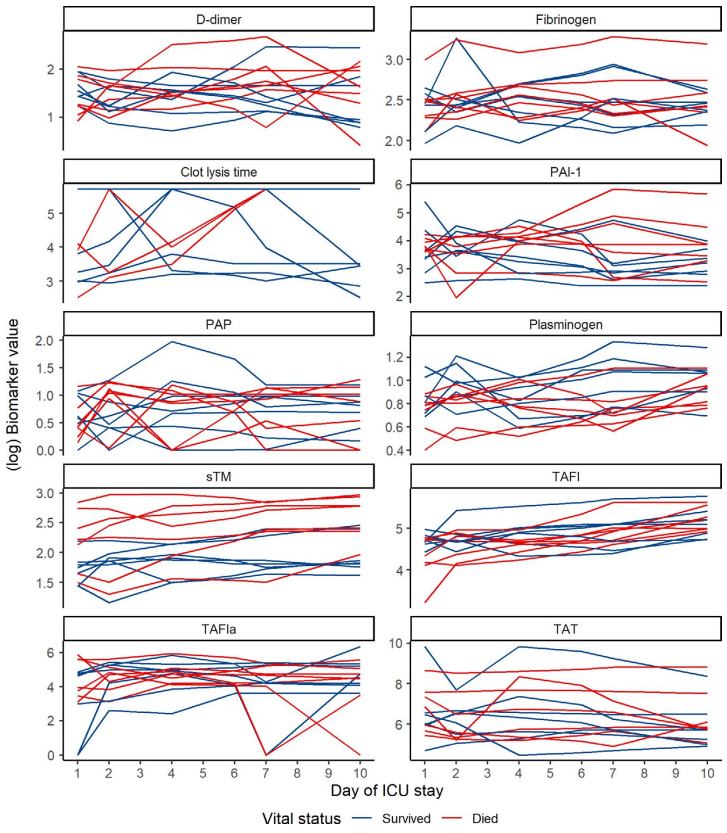


FIGURE 2 Individual biomarker trajectories of COVID-19(+) patients, stratified by survivors and nonsurvivors. COVID-19, coronavirus disease 19; PAI-1, plasminogen activator inhibitor 1; PAP, plasmin-antiplasmin complex; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated thrombin-activatable fibrinolysis inhibitor; sTM, soluble thrombomodulin; TAT, thrombin-antithrombin complex

and patient treatment outcomes]). To the best of our knowledge, our study using repeated measure modelling and unsupervised machine learning is the first of its kind to attempt to provide trajectory profiles of important biomarkers of coagulation and fibrinolysis in critically ill COVID-19(+) patients that are associated with death. Similar analyses on endotheliopathy were reported by Fraser et al.[35] using the same cohort. We report evidence of longitudinally increased coagulation, impaired fibrinolysis, and endothelial activation. Independent analyses of the various biomarkers suggest that there are differences in the baseline and time-dependent trajectories of plasminogen, PAI-1, sTM, and clot lysis times between survivors and nonsurvivors. These results are supported by our unsupervised longitudinal clustering algorithm, which similarly identified longer clot lysis times (i.e., inhibition of fibrinolysis), higher levels of PAI-1 and sTM, and lower levels of plasminogen in clusters of COVID-19(+) patients with higher mortality and signs of more severe disease.

sTM had the strongest longitudinal association with death in the ICU. Compared with those that survived, patients that died had elevated baseline and longitudinal sTM values. sTM levels increased for all COVID-19 patients during their ICU stay. These data corroborate our current understanding of COVID-19 disease pathophysiology that seemingly involves endothelial dysfunction, which is reflected by increased levels of (1) solubilized forms of membrane proteins such as thrombomodulin and syndecan-1 and (2) von Willebrand factor, which is stored in Weibel-Palade bodies of endothelial cells.[35]

Based on other reported studies that suggest inhibition of fibrinolysis,[20,25] we also hypothesize that inhibitors of fibrinolysis (such as PAI-1 and α_2-antiplasmin) are involved. The implication is that although coagulation (as demonstrated by elevated TATs) and

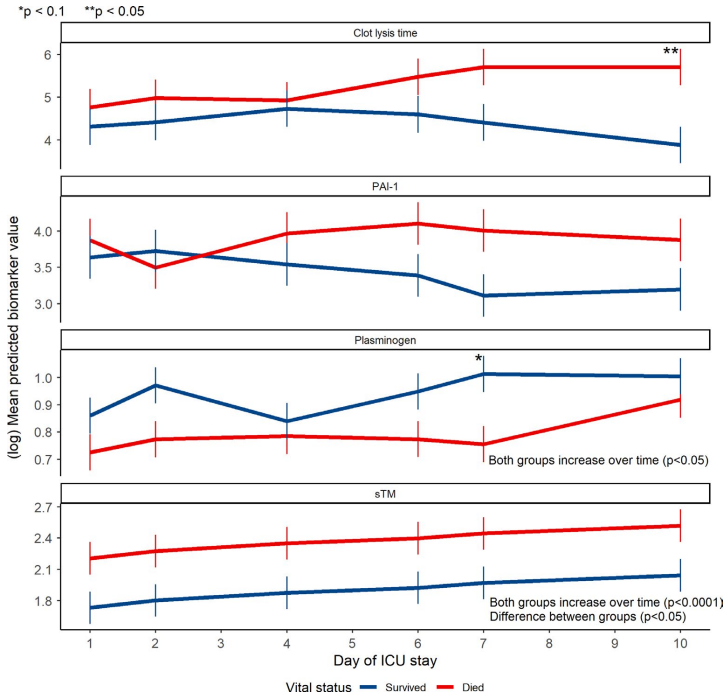
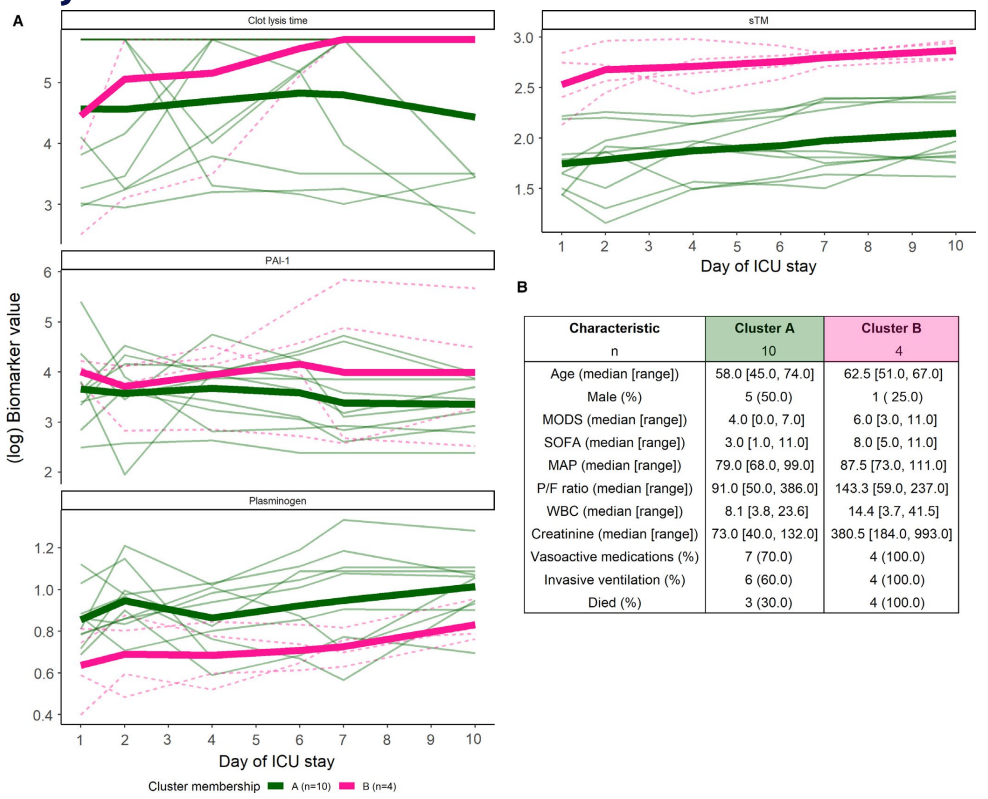


FIGURE 3 Mean predicted trajectories of select biomarker in COVID-19(+) survivors and nonsurvivors as determined by repeated measures linear mixed models. Error bars indicate standard errors. Differences between groups at individual time points were determined by interaction terms between day of measurement and vital status (*$p < .1$; **$p < .05$). Overall changes over time were determined by tests of fixed effects (ANOVA). The difference between groups for sTM was the main effect for vital status, with no interaction term included. COVID-19, coronavirus disease 19; PAI-1, plasminogen activator inhibitor 1; sTM, soluble thrombomodulin

fibrinolysis are both increased, inhibition of fibrinolysis tips the balance toward overall thrombosis. In our cohort of COVID-19(+) patients, we demonstrate that PAI-1 levels are elevated, and the values are higher in those with worse outcomes, although this difference did not reach statistical significance. This is in agreement with the genomic analyses performed by Gill et al.,[36] suggesting that transcription of *SERPINE1*, the gene for PAI-1, is up-regulated in this COVID-19(+) patient cohort compared with COVID-19(−) critically ill patients. Increased PAI-1 levels are consistent with our functional findings whereby overall fibrinolysis measured by clot lysis times is impaired, particularly in the nonsurvivors. The impairment of clot breakdown in COVID-19 could potentially reflect alterations in the clot structure because of components such as neutrophil extracellular traps or cell-free DNA,[50] which has been reported to possess antifibrinolytic properties.[51] Taken together with other factors that may promote coagulation such as endothelial dysfunction and inflammation,[35,52-54] platelet hyperreactivity,[14] and tissue factor expression in monocytes upon infection,[14,55] may all be involved in the overall thrombotic phenotype.

Other factors of fibrinolysis were investigated. The plasminogen level in the nonsurvivor group is 50% lower than that in the survivor group, suggesting consumption and/or activation of plasminogen may be involved in COVID-19, which likely generates elevated D-dimer levels. However, it is unclear why enhanced plasminogen activation (i.e., plasmin generation) would not be accompanied by elevated levels of the PAP complex. One explanation is that the PAP complex has a short half-life (~0.5 days), which fails to capture acute plasminogen consumption. Similar disparities were observed between TAFI and TAFIa. Levels of TAFIa not mirroring early consumption of TAFI may also be due to short functional half-life of TAFIa, which is ~7 min *in vivo*.[56] Another potential mechanism is that neutrophil-derived elastase is elevated in COVID-19 patients,[57,58] which was also observed in this same cohort.[34,36] Elastase modifies plasminogen to generate mini-plasminogen, a truncated form of plasminogen. Although activation of full-length or mini-plasminogen by tPA is comparable,[58] inhibition of activated mini-plasmin by α_2-antiplasmin is 100-fold slower than the full-length plasmin.[59,60] It is also possible that the PAP ELISA kit is not sensitive toward

A



B

Characteristic n	Cluster A 10	Cluster B 4
Age (median [range])	58.0 [45.0, 74.0]	62.5 [51.0, 67.0]
Male (%)	5 (50.0)	1 (25.0)
MODS (median [range])	4.0 [0.0, 7.0]	6.0 [3.0, 11.0]
SOFA (median [range])	3.0 [1.0, 11.0]	8.0 [5.0, 11.0]
MAP (median [range])	79.0 [68.0, 99.0]	87.5 [73.0, 111.0]
P/F ratio (median [range])	91.0 [50.0, 386.0]	143.3 [59.0, 237.0]
WBC (median [range])	8.1 [3.8, 23.6]	14.4 [3.7, 41.5]
Creatinine (median [range])	73.0 [40.0, 132.0]	380.5 [184.0, 993.0]
Vasoactive medications (%)	7 (70.0)	4 (100.0)
Invasive ventilation (%)	6 (60.0)	4 (100.0)
Died (%)	3 (30.0)	4 (100.0)

FIGURE 4 (A) COVID-19(+) patients clustered into two groups: cluster A (*green*, *solid line*) and cluster B (*pink*, *dashed line*) based on the selected biomarkers using a longitudinal k-means clustering algorithm. Mean (*bold lines*) and individual trajectories are displayed. (B) Select patient characteristics in each cluster. COVID-19, coronavirus disease 19; PAI-1, plasminogen activator inhibitor 1; sTM, soluble thrombomodulin

mini-plasmin-antiplasmin complex; however, this was not confirmed in this study.

Several studies to date have reported elevated levels of D-dimer in COVID-19(+) patients, particularly those with moderate to severe disease.[19,61,62] We confirmed elevated D-dimer levels in our cohort of COVID-19(+) patients, which were significantly higher than the levels in both COVID-19(–) critically ill patients and healthy controls. Although D-dimer was the single largest identifier of COVID-19(+) status, D-dimer was not associated with death. Our findings suggest that D-dimer lacks prognostic power to characterize the clinical course of patients with COVID-19, which is consistent with the apparent discrepancy between elevated levels and the thrombotic phenotype of COVID-19. These analyses also support earlier reports that suggest D-dimer testing upon hospital admission may not be a reliable predictor of thrombotic complications or treatment outcomes, demonstrated by a modest sensitivity and specificity of ~85%.[12,19] D-dimer level, however, was reported to decrease upon intensive prophylactic and therapeutic anticoagulation in COVID-19 patients, which correlated with reduced need for mechanical ventilation, increased gas exchange, and improved 30-day mortality outcome.[63,64] It is possible that with more rigorous and standardized reporting,[65] along with longitudinal monitoring, D-dimers may have more prognostic value.

Fibrinolytic factors such as plasminogen[66-68] and uPA[69] have been implicated in wound healing, whereas fibrinogen,[70,71] TAFI,[72-74] plasminogen,[75-77] and its receptors[78,79] have been implicated in inflammation. It is unclear how the altered fibrinolytic factor levels found in the critically ill COVID-19 patients would affect the clinical course and outcome by impacting systems beyond coagulation and fibrinolysis. Increasing the sample size will provide additional mechanistic details, whereas further defining the potential prognostic value of other fibrinolytic factors such as TAFI, which was excluded in our exploratory analyses but was approaching the *p* value cutoff ($p = .29$). Analyses of additional cohorts will also be needed to validate our initial findings.

There are limitations to this study. The patient population consisted of a small cohort recruited from a single ICU relatively early in the COVID-19 pandemic. This phase was characterized by concentrated outbreaks among vulnerable populations such as long-term care residents, and ICU care was not yet standardized among COVID-19 patients. This may be reflected in the unusually high mortality rate in this cohort. Therefore, it is unclear how our results will generalize to other patient populations. Furthermore, ELISAs may not be widely available because of cost, whereas carrying out clot lysis assays may require experienced technical staff, making quantification of these biomarkers challenging in a hospital setting. We attempted to address the small sample size by refraining from multivariable regression, and using a conservative p value cutoff for variable selection. Finally, although the results of this work have implications for the understanding of coagulopathy of COVID-19, we did not directly measure thrombotic outcomes. This will be the focus of future work.

Taken together, our findings improve the mechanistic understanding of COVID-19-associated coagulopathy. If validated, our findings have the potential to make direct impact in COVID-19 prognostication by identifying patients that are at greater risk of decompensation based on the joint trajectories of key biomarkers of fibrinolysis and endothelial dysfunction.

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CONFLICT OF INTEREST

COVID-BEACONS investigators include Paul Y. Kim, Calvin H. Yeh, Matthew Castelo, Bettina Hansen, Bernardo Trigatti, Jeffrey I. Weitz, Patricia C. Liaw, Alison Fox-Robichaud, Peter L. Gross, Geoff Werstuck, Colin A. Kretz, Keyvan Karkouti, Stuart McCluskey, and Claudia dos Santos. Dr. Weitz has received personal fees from Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Daiichi-Sankyo, Ionis Pharmaceuticals, Janssen, Merck, Novartis, Pfizer, and Portola, outside the submitted work. All other authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Douglas D. Fraser, Marat Slessarev, and Claudio Martin collected the patient samples and associated deidentified clinical data. Calvin H. Yeh, Scott McGilvray, Peter L. Gross, Patricia C. Liaw, Jeffrey I. Weitz, and Paul Y. Kim designed the experiments. Ganeem K. Juneja, Samantha E. Cerroni, James E. Chessum, Joel Abraham, Erblin Cani, and Douglas D. Fraser generated the data. Matthew Castelo, Bettina E. Hansen, and Paul Y. Kim analyzed the data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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3.2 Determining the biomarkers levels for the COVID- BEACONS study cohort

Only 21 patient samples for this cohort were analyzed so far and the overall mortality rate of that group was 61.1%. Baseline biomarker levels were measured in all the patients in this cohort, and the median and range were calculated (Table 5). The measured biomarker values were log-transformed and linear mixed effects models were used to compare trajectories of survivor and non-survivor patients in the ICU (Figure 4). The trajectories show the course of the biomarker levels over the days a patient spent in the ICU. Additionally, statistical analysis was done in order to see if any of the biomarkers were statistically different between the non-survivor and survivor groups (Table 6). Plasminogen levels were decreased in the survivor group ($p=0.0035$) and increased over time in the non-survivor group ($p=0.0292$). TAFI antigen levels decreased over time in the non-survivor group ($p=0.0201$), whereas fibrinogen levels increased over time ($p=0.0346$). On the other hand, D-dimer antigen levels had an inconsistent trajectory throughout the duration of patients' stay in the ICU. The other biomarkers were not significant at baseline, over time or between the survivor and non-survivor group.

TABLE 5: Median and range values of the baseline biomarker levels of the COVID-BEACONS study cohort.

Biomarker	Median [Range]
PAI-1 (ng/mL)	38.48 [2.14, 892.73]
Plasminogen (µg/mL)	62.48 [41.27, 212.84]
PAP (µg/mL)	860.50 [345.00, 16425.00]
TAFI (µg/mL)	13.63 [0.40, 33.43]
sTM (pg/mL)	6352.20 [3107.04, 13443.87]
D-Dimer (ng/mL)	168.19 [13.65, 1620.42]
Fibrinogen (mg/mL)	4.85 [1.41, 14.44]
TAT (ng/mL)	25.51 [2.76, 324.43]

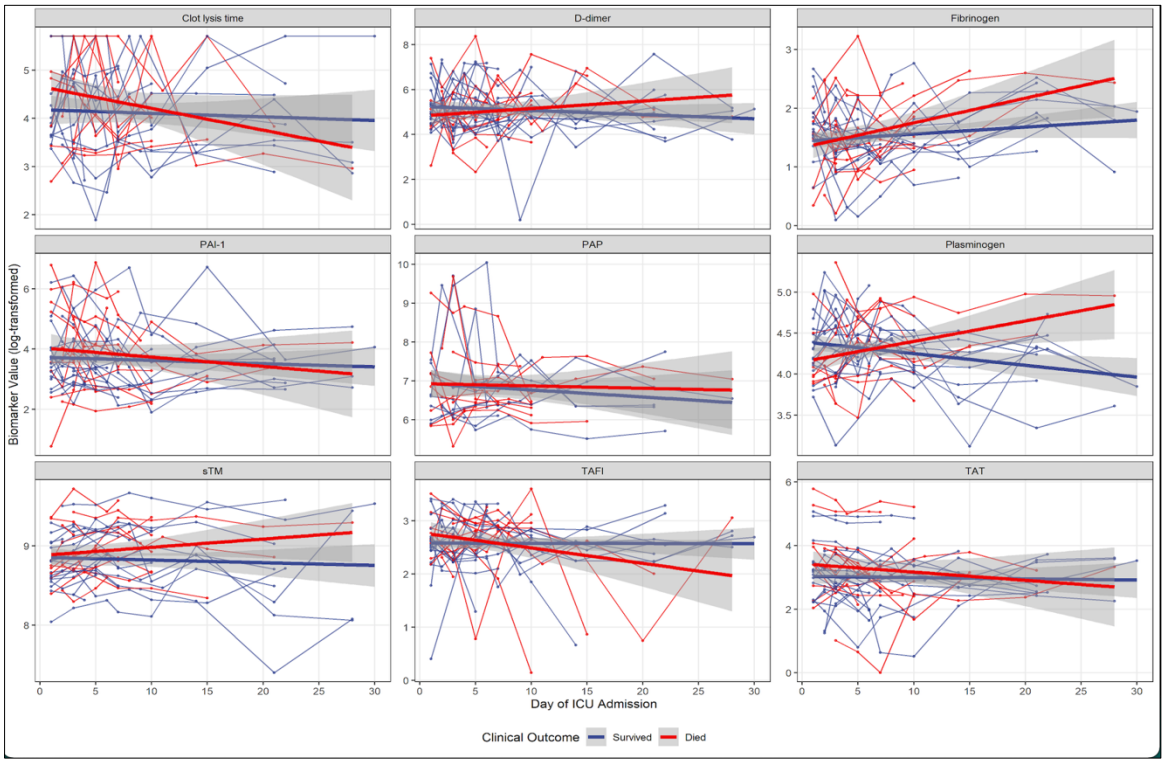

FIGURE 4: COVID BEACONS Study Cohort Biomarkers.

Individual longitudinal time courses are shown (*thin lines*). Data were stratified to survivors (*blue*) and non-survivors (*red*). Linear regression was then performed on the data (*thick lines*), with their 95% confidence interval (*grey*).

TABLE 6: Statistical analysis for significance in the biomarker levels between the survivors and non-survivors of the COVID-BEACONS study cohort. A p-value of less than 0.05 was considered statistically significant.

Biomarker	Significance
PAI-1	• No significance
Plasminogen	• Significant decrease over time in survived group (p=-0.0035) • Significant difference in trajectory with died group increasing over time (p=0.0292)
PAP	• No significance
TAFI	• Significant difference in trajectory with died group decreasing over time (p=0.0201)
sTM	• No significance
D-Dimer	• No significance
Fibrinogen	• Significant difference in trajectory with died group increasing over time (p=0.0346)
TAT	• No significance

3.3 Determining the biomarkers levels for the CanCOV study cohort

The biomarker values were log-transformed and linear mixed effects models were used to make longitudinal trajectories of the biomarkers of interest in the three patient groups (Figure 5). Among the 45 ICU patients, 24 (53%) died and there were no deaths in the other patient groups. Next, the biomarker values were log-transformed and linear mixed effects models were used to compare trajectories in ICU and ward patients compared to outpatients from date of symptom onset (Figure 6). D-dimer and sTM were significantly elevated for both hospitalized and ICU cohorts when compared with outpatients. PAI-1 was significantly elevated only in the ICU group between days 1 and 40. Plasminogen significantly decreased only in the ICU group from day 25 onwards. TAFIa increased over time only in the ICU cohort, with the levels being significant from day 35. Fibrinogen displayed similar trends as plasminogen whereby only the ICU was significantly decreased from day 25. A2AP, TAFI, and clot lysis times were not significantly different compared to COVID-19 outpatients over time.

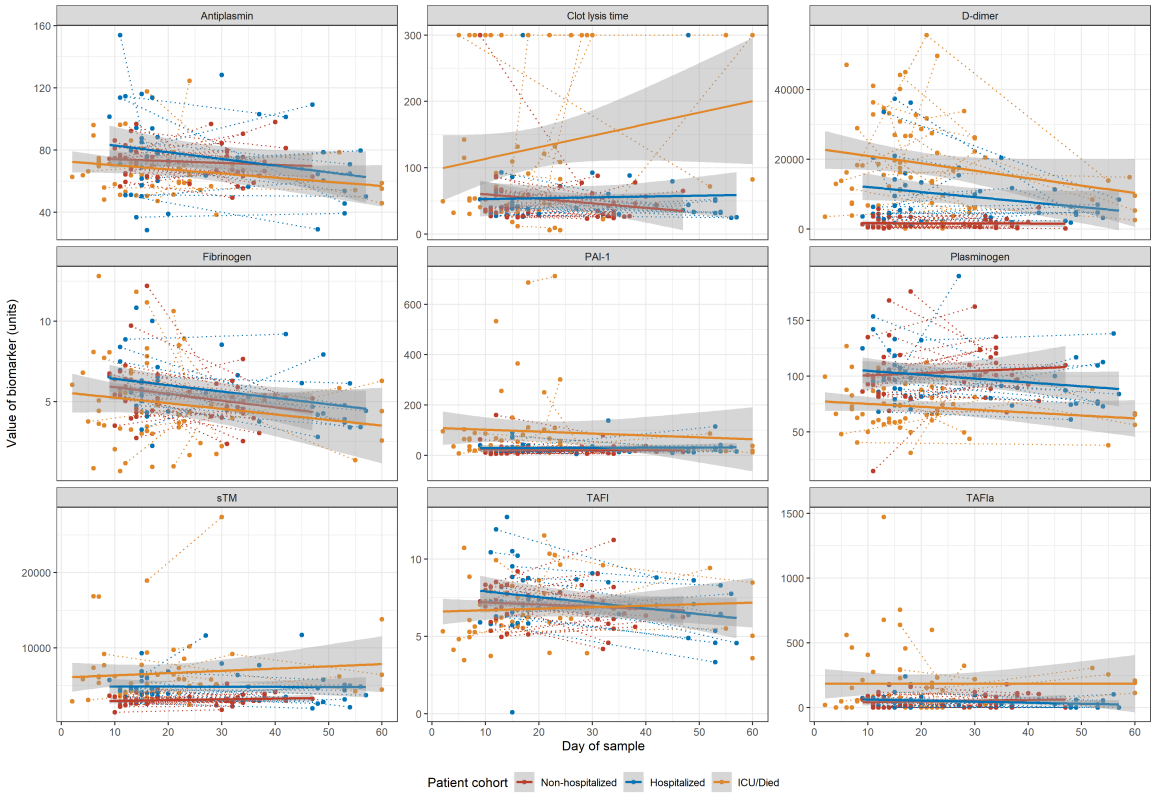

FIGURE 5: CanCOV Study Cohort Longitudnal Trajectories of the Biomarkers.

Individual log transformed longitudinal time courses are shown for the ICU patients *(yellow),* hospitalized/ward patients *(blue)*, and non-hospitalized/outpatients *(red).*

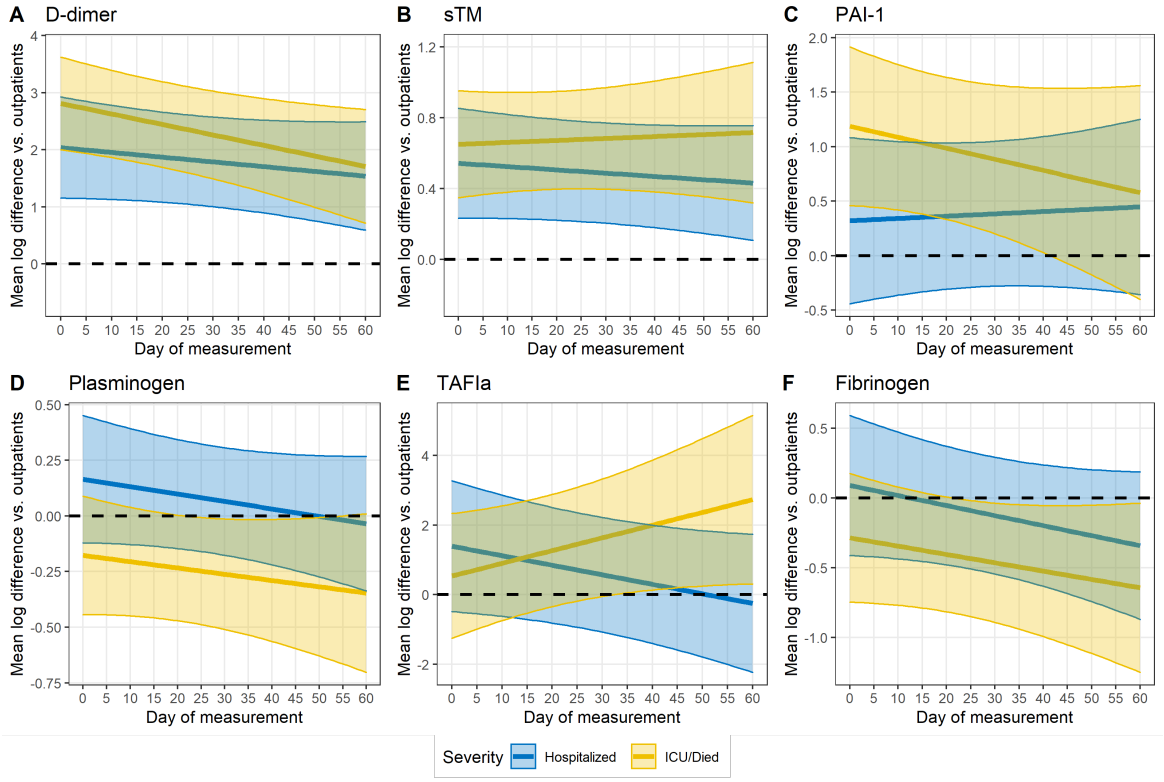

FIGURE 6: CanCOV study cohort biomarker trajectories stratified based on severity.

Mean log difference between the trajectory of biomarkers in COVID-19 positive patients, stratified by hospitalized *(blue)* and ICU/ died *(yellow)* versus outpatients *(dotted black line)*.

3.4 Determining the biomarkers levels for the ACTIV-4A trial cohort

For the ACTIV-4A trial cohort, the baseline biomarker levels were measured in all the patients, and the median and range was calculated (Table 7). In this cohort, the biomarker values were log transformed and linear effects models were used to compare the trajectories of the patients (Figure 7).

Additionally, statistical analysis was done in order to see if any of the biomarker levels were statistically different over the course of the disease in the patients. PAI-1 ($p=0.003$), sTM ($p<0.001$) and TAFIa ($p=0.003$) levels were increasing longitudinally, whereas fibrinogen ($p=0.042$) and A2AP ($p=0.028$) levels were decreasing over time. The other biomarkers were not significantly elevated or decreased for this cohort (Table 8).

TABLE 7: Median and range values of the baseline biomarker levels of the ACTIV-4A trial cohort. The values of some biomarkers is missing for some patients, which is indicated in the table.

Biomarker	Median [Range]
PAI-1 (µg/mL)	74 [37, 117]
Plasminogen (µg/mL)	121 [97, 139]
TAFI (µg/mL)	8.3 [5.4, 10.2]
TAFIa (pM)	211 [51, 352]
Missing	1
sTM (pg/mL)	4262 [3535, 5836]
Clot lysis time (min)	80 [50, 108]
Missing	6
Alpha-2 antiplasmin (µg/mL)	71 (58, 81)
Missing	1
Fibrinogen (mg/mL)	7.6 [4.8, 9.6]
TAT (ng/mL)	12 [6, 30]

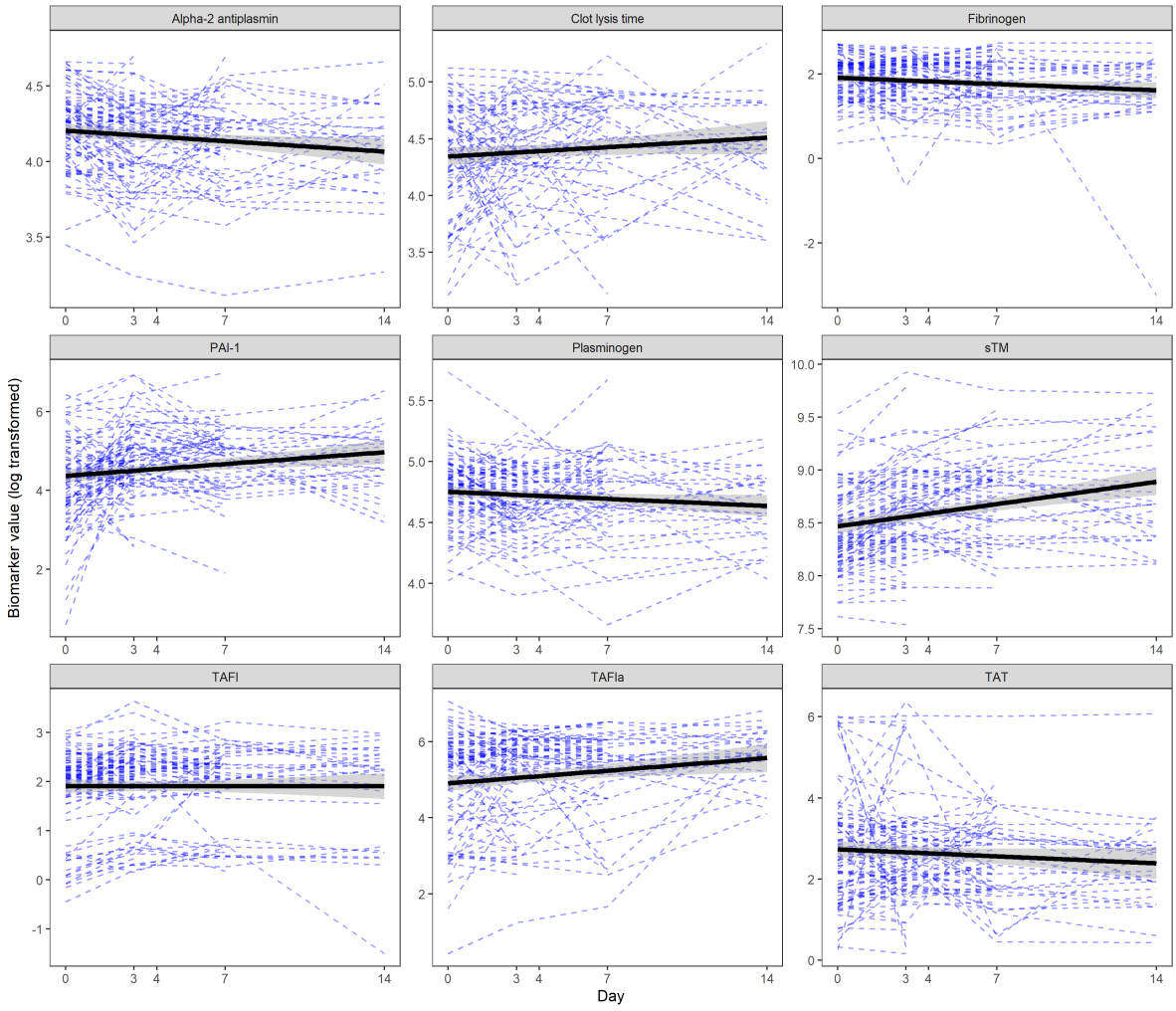

FIGURE 7: ACTIV-4A trial cohort biomarkers trajectories.

Individual log transformed longitudinal time courses are shown (*thin lines*). Linear regression was then performed on the data (*thick lines*).

TABLE 8: Statistical analysis for significance in the biomarker levels over the course of the disease in the ACTIV-4A trial cohort. The values of some biomarkers is missing for some patients, which is indicated in the table.

Biomarker	Mean log change per day	Standard error	p-value	Significant
PAI-1	0.044	0.015	0.003	Yes
Plasminogen	-0.005	0.003	0.119	
sTM	0.009	0.007	0.236	
TAFI	0.016	0.003	<0.001	Yes
Alpha-2 antiplasmin	-0.006	0.003	0.028	Yes
Fibrinogen	-0.020	0.010	0.042	Yes
TAT	-0.021	0.015	0.155	
Clot lysis time	0.010	0.006	0.082	
TAFIa	0.042	0.014	0.003	Yes

CHAPTER 4.0: DISCUSSION

Our pilot study cohort demonstrated a longitudinal increase in coagulation, impaired fibrinolysis, and endothelial activation. Independent analyses of the various biomarkers suggest that there are differences in the baseline and time-dependent trajectories of plasminogen, PAI-1, sTM, and clot lysis times between survivors and non-survivors. Specifically, sTM had the strongest longitudinal association with death and compared with those that survived, patients that died had elevated baseline and longitudinal sTM values. This data corroborates the current understanding that COVID-19 disease causes endothelial dysfunction which is reflected by the increased levels of solubilized forms of membrane proteins such as thrombomodulin (Fraser et al., 2020). We also demonstrated that PAI-1 levels and clot lysis times were elevated, and the values were higher in those with worse outcomes. The elevated levels of these two biomarkers suggests impaired fibrinolysis in COVID-19 patients which were also shown by Nougier et al. (Nougier et al., 2020). Increased coagulation and impairment of the fibrinolytic pathway was also demonstrated by the plasminogen levels which were 50% lower in the non-survivor group than that in the survivor group. This has been reported by Della-Morte and collogues, where they showed that lower levels of plasminogen increased the risk of mortality due to increased blood clots in their cohort of COVID-19 patients (Della-Morte et al., 2021). Several studies to date have reported elevated levels of D-dimer in COVID-19 patients, particularly those with moderate to severe disease (Huang et al., 2020; Wu & McGoogan, 2020; Yu et al., 2020). We confirmed elevated D-dimer levels in our cohort of COVID-19(+) patients, which were significantly higher than the levels in both COVID-19(−) critically ill patients and healthy controls. Although D-dimer was the single largest identifier of COVID-19(+)

status, D-dimer was not associated with death. Therefore, this cohort demonstrated that antigen levels of sTM, PAI-1, plasminogen, and clot lysis times have predictive ability for mortality in patients with COVID-19.

To validate our pilot cohort, we used the COVID-BEACONS, CanCOV and the ACTIV-4A trial cohorts. In the COVID-BEACONS study cohort, we observed that the COVID-19(+) patients with worse outcomes exhibited increased plasminogen and fibrinogen levels, and decreased TAFI levels. The elevated plasminogen levels are contrary to our pilot cohort and other literature, which has shown lower plasminogen levels being associated with worse outcomes (Della-Morte et al., 2021; Juneja et al., 2021). The high levels of plasminogen could be due to the fact that the samples for the pilot cohort were collected during the first wave of the pandemic. Whereas the samples for COVID-BEACONS cohort were collected during the third and fourth wave of the pandemic, by which time there were several different variants of the virus. Another difference can be due to the two different demographics of the cities (London and Hamilton) where the samples were collected. The elevated plasminogen levels and its association with worse outcomes has been attributed to plasmin(ogen) enhancing the virulence and infectivity of the SARS-CoV-2 virus by cleaving its S protein (Ji et al., 2020). The COVID-BEACONS study cohort also demonstrated elevated levels of fibrinogen which suggests a hypercoagulable state in patients with worse outcomes; this has been reported in COVID-19 patients with poor prognosis among hospitalized patients (Di Micco et al., 2020). The elevated fibrinogen levels have also been implicated as a response to inflammation (Luyendyk et al., 2019). We also reported lower levels of TAFI in the non-survivor patient group in this cohort. The

decreased TAFI levels suggest an increase in fibrinolysis in COVID-19 patients with worse outcomes, which is reflected by decreased levels of plasmin-antiplasmin complex (Tang et al., 2020). The COVID-BEACONS cohort showed an overall increase in coagulation and fibrinolysis, and no impairment of the fibrinolytic pathway or endothelial dysfunction. As mentioned previously, this could be due to the different demographics and variants of the pilot study. Additionally, interpretations may change once all the data for the 60 patients of this cohort is statistically analyzed and normalized with the clinical course timing and symptom onset. Lastly, this cohort showed that plasminogen, fibrinogen and TAFI levels hold prognostic value in COVID-19 disease outcome.

The third cohort we explored was the CanCOV study cohort. This cohort was divided into three different patient groups, and baseline biomarker levels were measured (Appendix: Table 1). This cohort differed from our pilot cohort because hospitalization (ICU and ward patients) was the measure of worse outcomes instead of mortality. Due to this, these two cohorts cannot be directly compared. However, this cohort also demonstrated that patients with worse outcomes had increased coagulation, impaired fibrinolysis and endothelial dysfunction. Similar to the pilot cohort, the hospitalized (ICU and ward patients) groups in the CanCOV study cohort had elevated sTM, PAI-1 levels and lower plasminogen levels. This validates that COVID-19 causes endothelial dysfunction and impaired fibrinolysis in patients and validates sTM, PAI-1 and plasminogen as prognostic biomarkers. Additionally, the hospitalized group in this cohort also had elevated D-dimer and TAFIa levels, which taken together with lower levels of plasminogen corroborates the hypercoagulable state that is observed in COVID-19 patients with worse

outcomes (Della-Morte et al., 2021; Fogarty et al., 2020; Huang et al., 2020; Ranucci et al., 2020; Wu & McGoogan, 2020; Yu et al., 2020). Longitudinally decreasing levels of fibrinogen were also observed in the hospitalized groups in this cohort. This is contrary to what was seen in the COVID-BEACONS study cohort. The lower levels of fibrinogen observed in this cohort could be explained by the protective effect of fibrinogen in COVID-19 and other inflammatory states (Tang et al., 2020; Thachil, 2020). It has been suggested that if the inflammatory condition stays unabated, the hemostatic system responds by causing widespread thrombi to limit dissemination of the harmful microbes or damage-associated proteins. These increased thrombi result in increased D-dimer levels as well as exhausted platelet granules which no longer release fibrinogen (Thachil, 2020). Therefore, the fibrinogen levels start decreasing while the D-dimer levels are increasing, which is observed in this cohort. Additionally, lower fibrinogen levels have also been associated with increasing pulmonary occlusion rate, therefore resulting in a worse outcome (Kucher et al., 2003). Overall, this cohort suggests that sTM, D-dimer, PAI-1, TAFIa, fibrinogen and plasminogen have the potential to be predictive of a worse COVID-19 outcome.

Lastly, the ACTIV-4A trial cohort was investigated. Initially, the ACTIV-4A trial investigated the role of using therapeutic doses of heparin in COVID-19 patients in order to improve outcomes. The trial did not improve the outcomes of critically-ill COVID-19 ICU patients, however, it showed success in the COVID-19 ward patients. The samples our study looked at were the COVID-19 outpatients who received therapeutic doses of heparin. This makes this cohort very different from our pilot cohort where all the patients were in the ICU. However, the baseline data and the biomarker trajectories from this cohort could

be used to validate the prognostic value of the biomarkers of interest. The COVID-19 patients in this cohort had longitudinally increased levels of PAI-1, sTM and TAFIa, which validates what has been seen in the past cohorts. The increased levels of these biomarkers also validate the hypercoagulable state, impaired fibrinolysis and endothelial dysfunction that is seen in patients with COVID-19. Similar to the CanCOV study cohort, this cohort also had longitudinally decreasing levels of fibrinogen which validates the protective response of fibrinogen to inflammation due to the SARS-CoV-2 virus (Thachil, 2020). This cohort also showed a decrease of A2AP levels over the course of the COVID-19 disease. This has been shown by Hammer & collogues and suggests that there is an increase in fibrinolysis in COVID-19 patients (Hammer et al., 2021). This cohort demonstrated that PAI-1, sTM, TAFIa, fibrinogen, and A2AP levels are potential prognostic biomarkers for COVID-19 disease.

CHAPTER 5.0: CONCULSION

Currently there is not enough literature on prognostic biomarkers for COVID-19 and some of it is conflicting in terms of which biomarker is predictive of COVID-19 disease severity, as well as if the levels of said biomarker should be increased or decreased. Therefore, more research needs to be done to identify these biomarkers which could prove to be useful predictors of COVID-19 disease outcome.

Our study has the potential to improve mechanistic understanding of COVID-19-associated coagulopathy. If validated, our findings (summarized in Appendix: Table 2) have the potential to make direct impact in COVID-19 prognostication by identifying patients that are at greater risk of decompensation based on the joint trajectories of key biomarkers of fibrinolysis and endothelial dysfunction. This can help us prevent mortality and also help promote and/or adjust therapies for patients with COVID-19, therefore providing a better outcome for these patients.

CHAPTER 6.0: FUTURE DIRECTIONS

In this study we investigated if biomarkers of coagulation, fibrinolysis and endothelial dysfunction hold prognostic value in COVID-19 disease severity. In order to validate and make this study robust, additional cohorts and patients should be added to it. Furthermore, other biomarkers, such as VWF, ADAMTS13, EPCR, cfDNA, P-selectin, and so on can also be quantified. These additional biomarkers can help provide more predictive studies and outcomes for this study. Additionally, all the data for the different cohorts needs to be normalized using the clinical data from date of symptom onset and analyzed similar to our pilot study (COVID-19 status; survivors vs non-survivors) in order to validate our initial findings.

Another complication that has been seen in patients infected with COVID-19 is that they can experience long-term effects due to the infection. This is known as post-COVID conditions or long COVID. Future experiments investigating this condition can help give insight into what results in this long-term condition (*i.e.* worse outcome) and explore any long-term functional changes due to the disease.

Platelets have recently been shown to exhibit hyperreactivity in response to infection, with increased circulating platelet-neutrophil and platelet-monocyte aggregates in severe patients. One of the experiments I am currently working on is isolating platelets from COVID-19(+) patients from the ICU and adding them back into plasma isolated from COVID-19(+) patients or normal human plasma, subsequently subjecting them a clot lysis experiment. In addition, we will perform similar experiments on critically-ill septic patients that are COVID-19(-) to identify whether COVID-19-specific parameters can be identified.

This experiment can give us great insight as to if platelets play a role in the impairment of the fibrinolytic system in COVID-19 patients.

Lastly, once all these biomarkers and cohorts are validated, we can move on to other disease state models and use the same study design in order to predict clinical course and outcome.

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CHAPTER 8.0: APPENDIX

TABLE 1: Summary of the baseline biomarker levels of the different patient groups in the CanCOV patients. Baseline biomarker values were averaged and categorized. Normal biomarkers levels are marked in green, low biomarker levels are in blue, and high biomarker levels are marked in red.

	CanCOV ICU Patients	CanCOV Ward Patients	CanCOV Outpatients
Total #	45	31	23
Mortality	24	0	0
PAI-1 (ng/mL)	45.54	22.85	17.94
Plasminogen (µM)	0.79	1.05	1.06
TAFI (nM)	111.21	125	125.34
TAFIa (pM)	352.64	46.34	46.54
sTM (ng/mL)	4.91	4.10	3.01
D-dimer (µg/mL)	20.07	11.60	1.71
Fibrinogen (mg/mL)	4.48	6.17	5.82
A2AP (µg/mL)	68.21	74.19	73.65
Lysis Times (min)	71.08	40.09	38.85

TABLE 2: : Summary of the baseline biomarker levels of ICU patients in all the cohorts. Baseline biomarker values were averaged and categorized. Normal biomarkers levels are marked in green, low biomarker levels are in blue, and high biomarker levels are marked in red.

	London Healthy Patients	London COVID-19(-)	London COVID-19(-)	COVID-BEACONS	CanCOV-ICU	ACTIV-4
Total #	14	14	14	21	45	91
Mortality	0	0	7	11	24	19
PAI-1 (ng/mL)	9.8	52.3	40.9	38.48	45.54	74
Plasminogen (µM)	1.8	1.3	1.2	0.68	0.79	1.32
PAP (µg/mL)	0.8	0.6	0.7	0.86	-	-
TAFI (nM)	129.8	121.3	106.9	235	111.21	143.1
TAFIa (pM)	26.6	162.3	75.9	224.59	352.64	211
sTM (ng/mL)	3.3	5.7	5.1	6.35	4.91	4.26
D-dimer (µg/mL)	0.7	1.3	3.5	0.17	-	1.234
Fibrinogen (mg/mL)	7.1	10.3	10.7	4.85	4.48	7.6
A2AP (µg/mL)	-	-	-		68.21	71
TAT(µg/mL)	9.3	30.9	54.2	25.51	-	12
Lysis Times (min)	17.26	133.07	192.29	121.20	71.08	80